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(54) Title: MASS PRODUCTION METHOD OF ANTIMICROBIAL PEPTIDE AND DNA CONSTRUCT AND EXPRESSION SYSTEM THEREOF

(57) **Abstract**

The present invention relates to DNA constructs that can produce antimicrobial materials efficientely from microorganisms and the preparation method thereof. The present invention also relates to the useful vector for the DNA construct. The DNA construct according to the present invention comprises a first gene coding for entire, a part of or a derivative of *purF* gene and a second gene coding for antimicrobial peptide. According to the present invention, antimicrobial peptides can be mass-produced by the following steps: preparing an expression vector containing a DNA construct comprising a fisrt gene coding for an entire, a part of or a derivative of *purF* gene and a second gene coding for antimicrobial peptide; transforming the bacterial host cells with the above-mentioned vector; culturing the transformed cell to express the above-mentioned DNA construct; and recovering the above antimicrobial peptide.

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MASS PRODUCTION METHOD OF ANTIMICROBIAL PEPTIDE AND DNA CONSTRUCT AND EXPRESSION SYSTEM THEREOF

TECHNICAL FIELD AND BACKGROUND ART

The present invention relates to the recombinant DNA technology. The present invention also relates to the mass-production of antimicrobial materials from microorganisms and aDNA construct and vector system. Biologically active peptide (antimicrobial peptide hereinafter) has little chance to develop resistance since the antimicrobial peptides show activity by a mechanism that is totally different from that of conventional antibiotics which have a serious problem of developing resistance. Therefore, the antimicrobial peptides have a high industrial applicability in the fields of pharmaceutics and the food industry.

The main obstacle in the industrial use of the antimicrobial peptide, however, is the difficulty in economical mass-production of the antimicrobial peptides. For instance, the production of the antimicrobial peptides by chemical synthesis is not economical. Also, there have been attempts to produce antimicrobial peptides by genetic engineering using microorganisms, in this case, however, the expression levels of the antimicrobial peptides are very low.

US patent 5,206,154 provides a DNA construct which comprises a polypeptide gene which is capable of suppressing the bactericidal effect of cecropin, and a cecropin gene

fused to the polypeptide gene. An example of such polypeptide disclosed in the patent is the *araB* gene.

US patent 5,593,866 provides a method for a microbial production of a cationic antimicrobial peptide, wherein the cationic peptides is expressed as a fusion to an anionic peptide to avoid degradation by a bacterial protease.

DISCLOSURE OF THE INVENTION

The present invention provides a DNA construct to mass-produce a antimicrobial peptides. The present invention also provides a DNA construct that can produce and recover antimicrobial peptides effectively from microorganisms.

Also, the present invention provides gene multimers that can increase the efficiency of expression, separation and purification of desired peptides and the construction method of such construct.

Further, the present invention provides an expression vector to mass-produce antimicrobial peptides from microorganisms.

Further, the present invention provides a method to mass-produce antimicrobial peptides form microorganisms.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a nucleotide sequence coding for an antimicrobial peptide of the present

invention.

Figure 2 is a nucleotide sequence coding for a fusion partner.

Figure 3 is a scheme of a fusion method between the fusion partner and the MSI-344 gene by generating a sequence encoding producing CNBr cleavage site.

Figure 4 is a scheme of a fusion method between the fusion partner and the MSI-344 gene by generating a sequence encoding producing hydroxylamine cleavage site.

Figure 5 is a scheme of the construction of the transcriptionally fused multimer.

Figure 6 is a scheme of the construction of the pGNX2 vector.

Figure 7 is a scheme of the construction of the pT7K2.1 vector.

Figure 8 is a scheme of the construction of the pGNX3 vector.

Figure 9 is the pGNX4 vector.

Figure 10 is a schme of the costruction of the pGNX5 vector.

Figure 11 is a SDS-PAGE electrophoretic analysis of the lysates of the transformants expressing MSI-344 by an induction with lactose or IPTG.

Figure 12 is a SDS-PAGE electrophoretic analysis of MSI-344 expression with various vectors.

Figure 13a is a SDS-PAGE electrophoretic anaylysis of the lysates of the transformants expressing various antimicrobial peptides by induction with lactose.

Figure 13b is a SDS-PAGE electrophoretic analysis of the lysates of the transformants expressing various antimicrobial peptides by an induction with lactose.

Figures 14a, 14b, 14c and 14d are SDS-PAGE electrophoretic analyses of the lysates of the transformants expressing various antimicrobial peptides by an induction with lactose.

Figure 15 is a SDS-PAGE electrophoretic analysis of the lysates of the transformants expressing the monomer, dimer and tetramer of the fusion genes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a DNA construct for mass-producing antimicrobial peptides effectively in *E. coli* or other prokaryotes.

One of the essential conditions for mass production of the antimicrobial peptides from microorganisms is to efficiently neutralize the toxicity of the antimicrobial peptides against the microorganisms. To this end, the present invention provides a DNA construct in which a whole gene, partial or derivatives of the *purF* gene (glutamine pyrophosphoribosyl pyrophosphate amidotransferase; Genbank No.: X12423) (Tso et al., J. Biol. Chem., 257: 3525, 1982, Makaroff et al., J. Bio. Chem., 258: 10586, 1983) is fused as a fusion partner to the gene coding for antimicrobial peptides.

The derivatives of *purF* gene used as a fusion partner in the DNA construct according to the present invention allows mass-production of the antimicrobial peptides as a fused polypeptide with purF derivatives in *Escherichia coli* without killing the host cells.

Therefore, it is possible to mass-produce the desired antimicrobial peptides from the host microorganisms using a strong expression system since they are not lethal to the host cell. In the case of using a fusion partner according to the present invention to express peptides, it is possible to cleave and separate the antimicrobial peptides from the fusion protein by using a protease or other chemicals. To achieve this, for instance, it is possible to insert a DNA sequence between the fusion partner and antimicrobial peptide genes encoding the cleavage site for proteases such as Factor Xa or enterokinase or chemicals such as CNBr or hydroxylamine.

For instance, to provide a CNBr cleavage site, restriction enzyme site containing Met codon (ATG) with correct leading frame such as *Afl* III, *Bsm* I, *Bsp*H I, *Bsp*LU11 I, *Nco* I, *Nde* I, *Nsi* I, *Ppu*10 I, *Sph* I, *Sty* I, or their isoschizomers could be inserted into the 3 ' end of the fusion partner. It is possible to make in-frame fusion of the fusion partner and the gene coding for antimicrobial peptide by inserting the restriction enzyme site into the 5 end of the gene coding for antimicrobial peptide that produces a compatible end to the enzyme site of the fusion partner.

It is also possible to insert a DNA sequence coding for Asn-Gly between the fusion partner and antimicrobial peptide genes. For instance, two genes can be fused by the following method. After inserting a restriction enzyme or isoschizomer site containing an Asn codon with correct reading frame at the 3' end of the fusion partner, the fusion partner is cleaved by the enzyme. At the 5 ' end of the gene coding for antimicrobial

peptide, a restriction enzyme site containing a Gly codon with correct reading frame that produces a compatible or blunt end with the corresponding site of the fusion partner is inserted and cleaved with the corresponding enzyme. The two cleaved DNA fragments may be connected to produce the fused gene. The genetic construct according to the present invention may be inserted into the host cell by cloning into any kind of expression vector, that is conventionally used in this field such as plasmid, virus or other vehicles that can be used to insert or incorporate the structural genes.

The present invention relates to a multimer that can increase the expression level by increasing the copy number of the gene of the required product and which can be separated and purified conveniently and the preparation method thereof.

The multimer according to the present invention is constructed by the following units.

1) A first restriction enzyme site that can generate an initiation codon Met, 2) a structural gene, 3) a ribosome binding site (RBS), and 4) a second restriction enzyme site generating a cohesive end which can be in-frame fused to the cohesive end generated by the first restriction enzyme and which can generate the initiation codon. Here, the stop codon and the RBS of the structural gene may overlap by ca. 2 bp or may be separated as far as 500 bp. The distance between the RBS and the second restriction enzyme site that can generate the initiation codon may be ca. 5 to 30 bp. The 3 ' and 5' ends of the multimer may be cleaved by the first or second restriction enzyme, respectively.

The multimer according to the present invention may be prepared by a variety of techniques known in the field of genetic engineering. One of the examples of such preparation method is given below.

After cleaving the units of a gene given above by the first and second restriction enzymes, the cleaved units is connected to produce a mixture containing multimers that include each unit with the same direction and multimers that have more than one unit with reverse direction. Since the multimers that contain more than one unit with reverse direction will have the first or second restriction enzyme site regenerated at the connection site, the multimer mixture may be cleaved simultaneously by the first and second restriction enzymes and separated by agarose gel electrophoresis, for instance, to separate the multimers those have units with the same direction only. The multimer according to the present invention is a transcriptionally fused multimer. This means that the repeated genes are transcribed into a single mRNA, but the gene expression product is not connected. In other words, the multimer is translated into many copies of a single product. In the case of the conventional translationally fused multimer, the desired product is present as a concatemer in a single polynucleotide, and an additional cleavage process is necessary to obtain the desired active product. In case that the expression product is a fusion protein, it requires a greater amount of reagent to cleave only with lower efficiency when compared to the transcriptionally fused multimer. Compared to the translationally fused multimer, the expressed multimer of the present

invention does not require additional cleavage processes or in the case it requires cleavage processes such as fused proteins, the amount of the reagent for the cleavage may be reduced since the number of peptide bonds to be cleaved per mole of the fused peptide is relatively smaller than the translationally fused multimer.

The multimer of the present invention may increase the gene expression in the host cell, have advantages in cleaving and purifying the desired product, and express in the host more efficiently when compared to the monomer. The multimer and the preparation method thereof are not limited in preparing peptides or fusion peptides. It can be widely applicable in expressing the unfused or fused gene coding for enzymes, hormones and antimicrobial polypeptides in microorganism.

Therefore, it is desirable to produce the DNA construct of the present invention in the form of transcriptionally fused multimer. In the case of preparing the DNA construct of the present invention in the form of transcriptionally fused multimer, it is advantageous to cleave and purify the products, and the multimer may be expressed in the host more efficiently than the monomer.

The present invention also relates to the expression vector that may induce the expression of foreign genes by lactose which is more economical than IPTG.

The expression vector according to the present invention is composed of high copy number replication origin, strong promoter and structural gene, and does not include $lacI^q$ gene.

The replication origin may be colE1 or p15A in the present invention. Examples of the strong promoters include tac, trc, trp, T7Φ10, P_L, other inducible or constitutive promoters in the microorganisms. Additionally, a selection marker gene that may be used to select the transformants of the vector may be included. These marker genes include antibiotic resistant genes against antibiotics such as ampicillin, kanamycin, tetracyline and chloramphenicol, or the genes that complement the auxotrophy of the host. Gene expression using the expression vector according to the present invention can be induced efficiently by adding lactose instead of IPTG preferably by adding IPTG and lactose simultaneously.

As an example, after transforming the plasmid containing the structural gene into the host cells, transformants are primary-cultured for 5 to 18 hours at 30-37 °C in a culture medium that include 50-300 μg/ml kanamycin. Afterwards, they are diluted to 1% (v/v) in a fresh media and cultured at 30-37 °C. To induce the expression, 0.01 mM- 10 mM IPTG is added when the OD_{600} reaches 0.2-2 in case of IPTG induction, or 0.2 - 2 % lactose is added when the OD_{600} reaches 0.2 - 2, or at the time of inoculation in the case of lactose induction. IPTG and lactose can be used simultaneously with a significantly reduced amount of IPTG. Additionally, it is desirable to include a transcriptional terminator in the expression vector according to the present invention.

It is possible to obtain the expression product as an inclusion bodies using the expression vector of the present invention. This property is useful in producing a product

lethal to the host.

A vector containing a structural gene of the present invention may be transformed into microorganisms by using conventional methods used in the fields of the present invention. For instance, the transformation may be achieved by $CaCl_2$ method or by physical methods such as electrophoration or microinjection into prokaryotic cells such as *E. coli*. There is no specific limitation for the host. For instance *E. coli* strain may be selected form BL21(DE3), BLR(DE3), B834(DE3), AD494(DE3), JM109(DE3), HMS174(DE3), UT400(DE3) and UT5600(DE3). Culture medium could be selected from LB, M9, M9CA, and R according to the characteristics of the host or transformants cells. Growth factors may be added to the media depending on the host requirements.

LB medium (bacto-tryptone 10g/l, yeast extract 5 g/l, NaCl 10 g/l)

M9 medium ($Na_2PO_4 \cdot 7H_2O$ 12.8 g/l, KH_2PO_4 3.0 g/l, NaCl 0.5 g/l, NH_4Cl 1 g/l, glucose 4 g/l, $MgSO_4$ 2 mM, $CaCl_2$ 0.1 mM)

M9CA medium (M9 medium + 0.2 % casamino acid)

R medium (Reisenberg medium; KH_2PO4 13.3 g/l, $(NH_4)_2PO_4$ 4.0 g/l, citric acid 0.17 g/l, $MgSO_4 \cdot 7H_2O$ 0.22 g/l, glucose 20 g/l, trace element solution 10 ml/l)

Trace element solution (ferric citrate 7.3 g/l, $CoCl_2 \cdot 6H_2O$ 0.5 g/l, $MnCl_2 \cdot 4H_2O$ 3.2 g/l, $CuCl_2 \cdot 2H_2O$ 0.3 g/l, H_3BO_3 0.7 g/l, $NaMoO_4 \cdot 2H_2O$ 1.68 g/l, Thiamin HCl 0.5 g/l, EDTA 1 g/l)

The invention will be further illustrated in detail by the following examples. It will be

apparent to those having conventional knowledge in this field that these examples are given only to explain the present invention more clearly, but the invention is not limited to the examples given below.

EXAMPLE 1. Preparation of a gene coding for an antimicrobial peptide

Two different MSI-344 genes were synthesized by the PCR method to express MSI-344 gene efficiently in *E. coli* and to ease the gene manipulation (Figure 1). Template for PCR was pNH18a-MBP-MSI-78 described in Korean patent application 97-29426. Sequence (a) was synthesized using primers No. 1 and No. 2 in Table 1 which was designed to separate MSI-344 by CNBr cleavage from the fusion peptide, and sequence (b) was synthesized using primers No. 3 and No. 4 in Table 1 which was designed to be cleaved by hydroxylamine. To subclone MSI-344 gene with correct reading frame into the expression vector, *Nde*1 (Sequence (a)) and *Sma*I (Sequence (b)) sites were inserted in front of MSI-344 gene and stop codons TAA and TGA were inserted behind the MSI-344 gene. Also to construct the transcriptional multimer, a ribosome binding site that overlaps 1 base pair with the stop codon and *Ase* I site were inserted. These two MSI-344 genes were cloned into pCR2.1 vector (Invitrogen, USA) to prepare vector pCRMSI containing sequence (a) and vector pCRMSI' containing sequence (b).

The antimicrobial peptide genes in Figure 1 were prepared by annealing chemically synthesized oligonucleotides (Table 1) or by performing PCR after annealing. In the

case of Apidaecin I, Indolicidin, and Tachyplesin I, DNA sequence was based on the amino acid sequence of a peptide (Maloy and Kark, Peptide Science, 37: 105, 1995) and the gene was chemically synthesized by using codons that can maximize the expression level in *E. coli*. In the case of Bombinin, CPF1, Drosocin, Melittin, HNP-I, PGQ, and XPF, the N- and C-terminal oligonucleotides which were designed to anneal to each other by 8-10 bp overlaps, were synthesized and the peptide gene was synthesized by PCR after annealing two oligonucleotides. The characteristics of each antimicrobial peptide are listed in Table 2.

Table 1

Sequences (5' ---> 3')		Primers
1	TCCGGATCCATATGGGTATCGGCAAATTC CTG	Primers for the synthesis of MSI-344 (32mer)
2	GCATTAATATATCTCCTTCATTACTTTTT CAGGATTTTAACG	Primers for the synthesis of MSI-344 (42mer)
3	GGATCCCGGGATCGGCAAATTCCTGAAAA AGG	Primers for the synthesis of MSI-344 (32mer)
4	GGATCCATTAATATATCTCCTTCATTAC	Primers for the synthesis of MSI-344 (28mer)
5	GGTAACAACCGTCCGGTTTACATCCCGCA GCCGCGTCCGCCGCACCCGCGTACTTGA	Primers for the synthesis of Apidaecin I (57mer)
6	AATTCTCAAGTACGCGGGTGCGGCGGACG CGGCTGCGGGATGTAAACCGGACGGTTGT TACC	Primers for the synthesis of Apidaecin I (62mer)
7	GGTATCGGTGCGCTGTCTGCGAAAGGTGC GCTGAAAGGTCTGGCGAAA	Primers for the synthesis of Bombinin (48mer)
8	CGAATTCTCAGTTCGCGAAGTGTTGCGCC AGACCTTTCGCCAGACCTTTCAGCGCACC	Primers for the synthesis of Bombinin (58mer)
9	GGTTTCGCGTCTTTCCTGGGTAAAGCGCT GAAAGCGGCGCTGAAAATC	Primers for the synthesis of CPF (50mer)
10	CGAATTCTCACTGCTGCGGCGCACCACCC AGCGCGTTCGCACCGATTTTCAGCGCCGC	Primers for the synthesis of CPF (60mer)

	TT	
11	GGTAAACCGCGTCCGTACTCTCCGCGTCCGACCTCTCAC	Primers for the synthesis of Drosocin (39mer)
12	CGAATTCTCAAACCGCGATCGGACGCGGGTGAGAGGTCGGACGCGGAGA	Primers for the synthesis of Drosocin (49mer)
13	GCATGCCATGGCGTGCTACTGCCGTATCCCGGCGTGCATCGCGGGTGAACGTCGTTACGG	Primers for the synthesis of HNP-1 (60mer)
14	CGAATTCTCAGCAGCAGAACGCCCACAGACGACCCTGGTAGATGCAGGTACCGTAACGAC	Primers for the synthesis of HNP-1 (60mer)
15	CATGATCCTGCCGTGGAAATGGCCGTGGTGGCCGTGGCGTCGTTGAG	Primers for the synthesis of Indolicidin (47mer)
16	AATTCTCAACGACGCCACGGCCACCACGGCCATTTCCACGGCAGGAT	Primers for the synthesis of Indolicidin (47mer)
17	GGTATCGGTGCGGTTCTGAAAGTTCTGACCACCGGTCTGCCGGCGCTG	Primers for the synthesis of Melittin (48mer)
18	CGAATTCTCACTGCTGACGTTTACGTTTGATCCAAGAGATCAGCGCCGGCAGACCGGT	Primers for the synthesis of Melittin (58mer)
19	GGTGTTCTGTCTAACGTTATCGGTTACCTGAAAAAACTGGGTACC	Primers for the synthesis of PGQ (45mer)
20	CGAATTCTCACTGTTTCAGAACCGCGTTCAGCGCACCGGTACCCAGTTTTTTCAG	Primers for the synthesis of PGQ (55mer)
21	CATGAAATGGTGCTTCCGTGTTTGCTACCGTGGTATCTGCTACCGTCGTTGCCGTTGAG	Primers for the synthesis of Tachyplasin (59mer)
22	AATTCTCAACGGCAACGACGGTAGCAGATACCCCGGTAGCAAACACGGAAGCACCATTT	Primers for the synthesis of Tachyplasin (59mer)
23	GGTTGGGCGTCTAAAATCGGTCAGACCCTGGGTAAAATCGCGAAAGTT	Primers for the synthesis of XPF (48mer)
24	CGAATTCTCATTTCGGCTGGATCAGTTCTTTCAGACCAACTTTCGCGATTTTACCCAG	Primers for the synthesis of XPF (58mer)
25	GGATCCATATGTGCGGTATTGTCGGTATCG	Primers for the synthesis of F (30mer)
26	CATATGGCGAGCTTCAAATACATCG	Primers for the synthesis of F (25mer)
27	GGATCCATATGTGCGGTATTGTCGGTATCG	Primers for the synthesis of F' (30mer)
28	GGATCCAATATTAGCTTCAAATACATCGCTC	Primers for the synthesis of F' (31mer)
29	GGATCCATATGTGCGGTATTGTCGGTATCG	Primers for the synthesis of F3 (30mer)
30	GGATCCAATATTCGCATGCGCAGCTTCAAATACATCG	Primers for the synthesis of F3 (HA) (37mer)
31	CGGGATCCACATGTGGCGAGCTTCAAATAC	Primers for the synthesis of F3 (CB) (30mer)
32	GGATCCATATGTGCGGTATTGTCGGTATCG	Primers for the synthesis of F4 (30mer)
33	GCGGATCCACATGTCGGCTTCCAG	Primers for the synthesis of F4 (CB) (31mer)

34	AATATTGTCGGCTTCCAGCGGGTAG	Primers for the synthesis of F3 (HA) (25mer)
35	CATATGCTTGCTGAAATCAAAGG	Primers for the synthesis of BF (23mer)
36	AATATTGCCAGCACCCTCCTGTCCTCGGTG	Primers for the synthesis of BF (30mer)
37	TTCGCTTGCGCGACCACT	Primers for purF G49A mutant (18mer)
38	TGCGAACGGGTGGAGCCGTTAGACTG	Primers for purF N102L mutant (26mer)
39	GCGGATCCAAGAGACAGGATGAGGATCGTTTCGC	Primers for the synthesis of kan^Rgene (34mer)
40	CGGATATCAAGCTTGGAAATGTTGAATACTCATACTCTTC	Primers for the synthesis of kan^Rgene (40mer)

Table 2

	Amino acid residue	Molecular weight (kDa)	Origin
Apidaecin I	18	2.11	Insect (A.mellifera)
Bombinin	24	2.29	Frog (B. variegata)
Cecropin A	36	3.89	Moth (H. cecropia)
CPF1	27	2.60	Frog (X. Laevis)
Drosocin	19	2.11	Fly (D.melanogaster)
HNP1	30	3.45	Human (alpha-defensin)
Indolicidin	13	1.91	Cow
MSI-344	22	2.48	Frog (X. laevis)
Melittin	26	2.85	Insect (H. cecropia)
PGQ	24	2.33	Frog (X. laevis)
Tachyplesin I	17	2.27	Crab (T.tridentatus)
XPF	25	2.64	Frog (X. laevis)

EXAMPLE 2. Preparation of fusion partner

To use as a fusion partner, *purF* derivatives shown in Figure 2 were obtained from the chromosomes of *E. coli* and *Bacillus subtilis* using PCR. The fusion partner F was prepared by CNBr cleavage, and F', F5 and BF by for hydroxylamine cleavage. F3 and F4 were prepared as two different forms; one for CNBr cleavage (F3(CB), F4(CB)), and

another for hydroxylamine cleavage (F3(HA), F4(HA), F4a(HA)). Fusion partners F, F', F3(HA), F3(CB), F4(HA), F4a(HA), F4a(CB), F5, BF are indicated in sequences No. 1 - 9, respectively.

1) *purF* derivative F

The derivative is a coding for 61 amino acid from the N-terminus of the *E. coli purF* protein (Figure 2). Nde I site including start codon Met was inserted at the 5' end, and *Nde* I site including Met codon that encodes cleavage site for CNBr was inserted at the 3' end.

2) *purF* derivative F'

To remove the internal hydroxylamine cleavage site, the 49th glycine residue (GGG) was substituted with alanine (GCG, see Figure 2) by site-directed mutagenesis using primer # 36 in Table 1, and *Ssp* I site containing AAT coding for asparagine was added after alanine codon (number 57) by PCR to form a hydroxylamine cleavage site.

3) *purF* derivative F3

The 49th glycine residue was substituted with alanine as in F'. Asparagine at the 58th residue was substituted with alanine and alanine-asparagine was added after the 59th histidine (F3(HA)). In case of F3 for CNBr cleavage (F3(CB)), a DNA sequence that codes for Met and includes *Bsp*LU11I site was added after histidine at the 59th residue.

4) *PurF* derivative F4

This derivative is composed of 159 amino acid residues from the N-terminus of the *purF*

protein. There exists two hydroxylamine sites in wild-type *purF* protein. To remove these sites, the 102nd asparagine codon (AAC) was substituted with leucine codon (CTC, underlined in Table 2) by site-directed mutagenesis with primer # 37 (Table 1) to form F4(HA). F4a(HA) was prepared by double substitution of the 49th glycine with alanine and the 102nd asparagine with leucine. In the case of F4(HA) and F4a(HA) for hydroxylamine cleavage, the *Ssp*I site including asparagine codon was added at the 3 ' end. In the case of F4a(CB) for CNBr cleavage, *Bsp*LU11 I site including Met codon was added at the 3' end.

5) *purF* derivative F5

This derivative composed of a sequence from the 60th methionine to the 148th aspartic acid of the *purF* protein, and *Ssp* I site was added at the 3' end.

6) *purF* derivative BF

BF is a *purF* derivative of *B. subtilis* and includes 43 amino acid residues and *Ssp* I site coding for Asn at the 3' end.

EXAMPLE 3. Preparation of DNA construct coding for fused peptides

Among the peptide genes prepared in Example 1, the genes encoding peptide that contains glycine at the first amino acid were fused to fusion partners for the hydroxylamine cleavage, F4a(HA), F5 and BF. Other peptides (HNP-I, Indolicidin, Tachyplesin) were fused to the fusion partners for the CNBr cleavage, F, F3(CB) and

F4a(CB) (Table 3).

A method of fusion between the fusion partner and the gene coding for an antimicrobial peptide while producing the CNBr cleavage site (Met) or hydroxylamine cleavage site (Asn-Gly) is shown in Figures 3 and 4, respectively. In the case of fusion with fusion partner F for CNBr cleavage, the fusion partner and the MSI-344 gene were fused using the *Nde* I site to produce DNA construct FM (Figure 3a). In case of fusion with F3(CB) or F4(CB), the peptide genes are chemically synthesized and fused to 3' end *Bsp*LU11 I site of the fusion partner by complementary 5' *Nco* I site for HNP-I, and 5' *Bsp*Lu11 I site for indolicidin and tachyplesin, respectively.

The fusion with the fusion partner for hydroxylamine cleavage (F', F3(HA), F4(HA), F4a(HA), F5, BF) was carried out by cleaving the fusion partner with *Ssp* I and MSI-344 by *Sma* I, and connecting these DNA fragments to generate Asn-Gly site for the hydroxylamine cleavage. In the case of the genes for Apidaecin I, Bombinin, CPF1, Drosocin, Melittin, PGQ and XPF, it was not necessary to digest with restriction enzyme before the fusion with the fusion partner cleaved with *Ssp* I, since they have 5' blunt ends.

EXAMPLE 4. Preparation of transcriptionally fused multimer

A monomeric unit that can produce multimers was constructed consisting of *Nde* I site coding for Met, structural gene, RBS, and *Ase* I site that connects with *Nde* I to generate

Met. As structural genes, F4a(HA)-MS I344 fusion gene (F4Ma) and F5-MSI344 fusion gene (F5M) were used. The monomeric units were digested with *Nde* I and *Ase* I, and the isolated monomeric units were reconnected. Obtained DNA fragments were digested again with *Nde* I and *Ase* I, and the multimers were separated by agarose gel electrophoreses. By using this method, monomer (F4Ma), dimer (F4MaX2) and tetramer (F4MaX4) of F4Ma and monomer (F5M), dimer (Fm5MX2) and tetramer (F5MX4) of F5M were obtained.

EXAMPLE 5. Expression vector

To express foreign gene in *E. coli*, two expression vectors pGNX2 and pT7K2.1 were constructed by using T7Φ10 promoter, high copy number replication origin (colEI of pUC family), and kanamycin resistance gene. To construct pGNX2, *bla* gene in commercially available pUC19 (ampicillin resistance gene: Amp^R) was substituted with kanamycin resistance gene (Kan^R). To this end, pUC19 was digested with *Ssp* I and *Dra* I to separate 1748 bp DNA fragment having 1748 bp, and Kan^R gene was amplified by PCR by using Tn5 of *E. coli* as a template and primers # 39 and # 40 (Table 1). The PCR product was digested with BamH I and Hind III, filled-in by Klenow treatment, and cloned int pUC19 digested with *Ssp* I and *Dra* I, resulting in pUCK2. After this vector was digested with *Nde* I and filled in by Klenow treatment, it was religated to contruct pUCK2ΔNdel. The final plasmid pGNX2 was constructed by cloning the fragment

containing T7Φ10 promoter and RBS from pT7-7 (USB, USA) that was digested with *Bam*H I, filled-in by Klenow treatment, and then digested with *Ase* I, into the pUCK2ΔNdeI vector that was digested with *Hind* III, filld-in by Klenow treatment, and then digested with *Ase* I. T7Φ10 promoter and kanamycin resistant gene (Kan^R) are oriented to the same direction in pGNX2 (Figure 6).

To construct the plasmid pT7K2.1, the bla gene was removed from pT7-7 by digestion with SspI and BgI I, and the following treatment with T4 DNA polymerase to make blunt ends. Kan^R gene was prepared as in pGNX2 and the two DNA fragments were ligated to construct pT7K2. Final plasmid pT7K2.1 was constructed by removing *Ase* I site from this vector (Figure 7). *E. coli* HMS174 (DE3) transformed with pGNX2 was deposited to Korean Collection of Type Cultures (KCTC) in Korea Research Institute of Bioscience and Biotechnology located at Yusong-gu Eun-dong, Taejon, Korea on May 29, 1998 and the number KCTC0486BP was given. To construct pGNX3, pGNX2F4M was partially digested with *Bsp*H I, and the fragment that has a cut in a single *Bsp*H I site was separated and further digessted with *Bam*H I. To prepare fragment containing T7 and rmBT1T2 terminators, 132 bp fragment from pET11a digested with *Bam*H I and *Eco*R V and a 488 bp fragment from ptrc99a digested with *Bam*H I and *Eco*R V were ligated. These fused fragments were cleaved by *Bam*H I and *Bsp*H I, and cloned into the vector prepared as above to construct pGNX3F4M (Figure 8).

To prepare pGNX4, a 3052 bp fragment was isolated from pETACc digested with *Xba* I and *Alw*N I, and a 2405 bp vector fragment from the pGNX3F4M digested with *Xba* I and *Alw*N I resulting in pGNX4F4M (Figure 9).

To construct pGNX5, pGNX3F4M was partially digested with *Ase* I, then digested with *Xba* I, and treated with Klenow fragment. A fragment obtained from PCR-TrpPO digested with *Eco*R I and *Nde* I and then treated with Klenow fragment was cloned with the above vector fragment to construct pGNX5F4M (Figure 11).

EXAMPLE 6. Production of antimicrobial peptides

DNA constructs obtained by fusing the MSI-344 to fusion partners, F3, F4, F4a, F5 and BF, were cloned into pGNX2 digested with *Nde* I and *Bam*H I and pT7K2.1 digested with *Nde* I and *Bam*H I, respectively. In case F (entire *purF*) was used as the fusion partner, it was cloned into pET24a (Novagen, USA) digested with *Nde* I and *Xho* I. In case of a multimer, it was cloned into the *Nde* I site of pGNX2 and pT7K2.1. The genes coding for Apidaecin I, Indolicidin, Tachyplesin I, Bombinin, CPF1, Drosocin, Melittin, HNP-I, PGQ and XPF were fused to the fusion partner F4 and cloned into pGNX2 digested with *Nde* I and *Eco*RI. When F3 was used, *Bam*H I and *Eco*R I sites of pRSETc were used for cloning (Table 3).

The plasmids 2,3,4,5,6 and 7 in Table 3 were transformed into *E.coli* HMS174(DE3) by

using the $CaCl_2$ method. R medium supplemented with casamino acid was used as a culture medium, and the peptide expression was induced when OD_{600} was between 0.2 and 0.4 by adding 2 % lactose and 2mM IPTG, respectively. The expression level was quantified by scanning the results from SDS-PAGE by a densitometer and as the percent of fusion peptide in total cell proteins (Figure 11). In Figure 11, M represents molecular weight standard marker, and lanes 1 through 6 represent the expression from the transformants with plasmids 2,3,4,5,6,and 7 in Table 3 by lactose induction, and lanes 7 through 12 represent the expression from the transformants with plasmids 2,3,4,5,6,and 7 in Table 3 by IPTG induction. Lanes 13 and 14 represent the expression from the transformant with plasmid 43 (*E. coli purF*; EF) by lactose and IPTG induction, respectively. As in the same manner, MSI-344 was expressed using *E.coli* HMS174(DE3) transformed with plasmids 44, 45 and 46 in Table 3 and by lactose induction (Figure 12). It can be seen that the expression level is higher with the plasmid having transcriptional terminator. With the HMS174 (DE3) transformed with plasmid 4 in Table 3, the expression of fusion peptide was induced by lactose and cells were harvested 9 hours after induction. The cells were sonicated and precipitates were obtained by centrifugation. After dissolving the precipitates by placing for 2 hours at room temperature in solution containing 9 M urea, 20 mM potassium phosphate (pH 8.5), the sample was loaded onto SP-sepharose FF column (Pharmacia, Sweden), and the fusion

peptide F4Ma was eluted using 0.3 ~ 1.0 M NaCl. Purified F4Ma was reacted in 0.5 ~ 2 M hydroxylamine and 0.4 M potassium carbonate (pH 7.5-9.5) buffer to cleave MSI-344 from the fusion partner. After desalting, the reaction mixture was loaded onto SP sepharose FF column (Pharmacia, Sweden) again to elute MSI-344 with 0.4 ~ 1M NaCl. Purified MSI-344 was identified by HPLC, MALDI-MS and amino acid sequencing.

EXAMPLE 7.

Other plasmids in Table 3 were transformed into *E. coli* HMS174 (DE3) by $CaCl_2$ method. R medium supplemented with casamino acid was used as a culture medium, and the peptide expression was induced by adding 2 % lactose when OD_{600} was between 0.4 and 0.6. The expression level was quantified by scanning the results from SDS-PAGE by a densitometer and as the percent of fusion peptide in total cell proteins. The results of the expression of each antimicrobial peptide are shown in Figures 13a and 13b and Table 3. In Figure 13a, lanes 1 through 6 represent the results from the transformants with plasmids 10,12,15,20,21 and 23 in Table 3. In Figure 13b, lanes 1 through 9 represent the results from the transformants with plasmids 11,13,14,16,22,17,18,24 and 19 in Table 3. Figures 14a -14d represent the expression results of plasmids 25 - 42 in Table 3. Buforin IIbx2 and Buforin IIx4 are dimer and tetramer of Buforin IIb, respectively, and constructed as described in Example 4. The corresponding plasmids, systems and

expression results were indicated in parenthesis below:

Figure 14a: 1(25)

Figure 14b: 1(26), 2(31), 3(36)

Figure 14c: 1(27), 3(32), 3(37), 4(28), 5(33), 6(38), 7(29), 8(34), 9(39), 10(30), 11(35), 12(40)

Figure 14d: 1(41), 2(42), 3(43)

Table 3

Number	peptide	Fusion partner	Cleaving method	Cloning vector	Plasmid	strain	Expression rate (%)
1	MSI-344	F	CNBr	pET24a	PETFM	BL21(DE3) BLR(DE3)	9
2	MSI-344	F3	HA	pGNX2	pGNX2F3M	BL21(DE3) HMS174(DE3)	10
3	MSI-344	F4(HA)	HA	pGNX2	pGNX2F4M	BL21(DE3) HMS174(DE3) JM109(DE3) UT400(DE3) UT5600(DE3)	30
4	MSI-344	F4(HA)	HA	pGNX2	pGNX2F4Ma	BL21(DE3) HMS174(DE3) JM109(DE3) UT400(DE3) UT5600(DE3)	30
5	MSI-344	F4(HA)	HA	pT7K2.1	pT&KF4M	BL21(DE3) HMS174(DE3) JM109(DE3) UT400(DE3) UT5600(DE3)	30
6	MSI-344	F4(HA)a	HA	pT7K2.1	pT&KF4Ma	BL21(DE3) HMS174(DE3) JM109(DE3) UT400(DE3) UT5600(DE3)	30
7	MSI-344	F5	HA	pGNX2	pGNX2F5M	BL21(DE3) HMS174(DE3)	20
8	MSI-344	F5	HA	pT7K2.1	pT7KF5M	BL21(DE3) HMS174(DE3)	20
9	MSI-344	BF	HA	pGNX2	pGNX2BFM	BL21(DE3) HMS174(DE3)	12
10	Apidaecin I	F3	HA	pRSETc	pRF2Ap	BL21(DE3) pLysS	25
11	Apidaecin I	F4(HA)	HA	pGNX2	pGNX2F4Ap	BL21(DE3) pLysS	8.7
12	Bombinin	F3	HA	pRSETc	pRF3Bp	BL21(DE3) pLysS	23
13	Bombinin	F4(HA)	HA	pGNX2	pGNX2F4Ap	BL21(DE3) pLysS	33.6

14	CPF	F4(HA)	HA	pGNX2	pGNX2F4Cpf	BL21(DE3) pLysS	9.0
15	Drosocin	F3	HA	pRSETc	pRF3Dp	BL21(DE3) pLysS	14
16	Drosocin	F4(HA)	HA	pGNX2	pGNX2F4Dp	BL21(DE3) pLysS	25
17	Melittin	F4(HA)	HA	pGNX2	pGNX2F4Me1	BL21(DE3) pLysS	26
18	PGQ	F4(HA)	HA	pGNX2	pGNX2F4Pg	BL21(DE3) pLysS	20.2
19	XPF	F4(HA)	HA	pGNX2	pGNX2F4Xp	BL21(DE3) pLysS	26.5
20	HNP-I	F3	CNBr	pRSETc	pRF3Hp	BL21(DE3) pLysS	26.3
21	Indolicidin	F3	CNBr	pRSETc	pRF3Id	BL21(DE3) pLysS	29
22	Indolicidin	F4(CB)	CNBr	pGNX2	pGNX2F4Id	BL21(DE3) pLysS	20.7
23	Tachyplesin I	F3	CNBr	pRSETc	pRF3Tp	BL21(DE3) pLysS	30
24	Tachyplesin I	F4(CB)	CNBr	pGNX2	pGNX2F4Tp	BL21(DE3) pLysS	21.8
25	Buforin I	F4(HA)	HA	pGNX3	pGNX3F4BI	HMS174(DE3)	25
26	Buforin II	F4(HA)	HA	pGNX3	pGNX3F4BII	HMS174(DE3)	30
27	Buforin II	F5(HA)	HA	pGNX3	pGNX3F4BII	HMS174(DE3)	20
28	Buforin II	F5(HA)	HA	pGNX4	pGNX3F4BII	HMS174(DE3)	18
29	Buforin II	BF(HA)	HA	pGNX3	pGNX3F4BII	HMS174(DE3)	4
30	Buforin II	BF(HA)	HA	pGNX4	pGNX3F4BII	HMS174(DE3)	4
31	Buforin IIa	F4(HA)	HA	pGNX3	pGNX3F4BIIa	HMS174(DE3)	28
32	Buforin IIa	F5(HA)	HA	pGNX3	pGNX3F4BIIa	HMS174(DE3)	20
33	Buforin IIa	F5(HA)	HA	pGNX4	pGNX3F4BIIa	HMS174(DE3)	18
34	Buforin IIa	BF(HA)	HA	pGNX3	pGNX3F4BIIa	HMS174(DE3)	4
35	Buforin IIa	BF(HA)	HA	pGNX4	pGNX3F4BIIa	HMS174(DE3)	4

36	Buforin IIb	F4(HA)	HA	pGNX3	pGNX3F4B IIb	HMS174(DE3)	25
37	Buforin IIb	F5(HA)	HA	pGNX3	pGNX3F4B IIb	HMS174(DE3)	20
38	Buforin IIb	F5(HA)	HA	pGNX4	pGNX3F4B IIb	HMS174(DE3)	18
39	Buforin IIb	BF(HA)	HA	pGNX3	pGNX3F4B IIb	HMS174(DE3)	20
40	Buforin IIb	BF(HA)	HA	pGNX4	pGNX3F4B IIb	HMS174(DE3)	15
41	Buforin IIbx2	BF(HA)	HA	pGNX4	pGNX3F4B IIbx2	HMS174(DE3)	20
42	Buforin IIbx4	BF(HA)	HA	pGNX4	pGNX3F4B IIbx4	HMS174(DE3)	20
43	MSI-344	EF	HA	pGNX2	pGNX2EF M	HMS174(DE3)	30
44	MSI-344	F4(HA)	HA	pGNX3	pGNX3F4 M	HMS174(DE3)	35
45	MSI-344	F4(HA)	HA	pGNX4	pGNX4F4 M	HMS174(DE3)	35
46	MSI-344	F4(HA)	HA	pGNX5	pGNX5F4 M	HMS174(DE3)	15

EXAMPLE 8.

The constructs prepared in Example 4, such as monomer (F4Ma), dimer (F4MaX2) and tetramer (F4MaX4) of F4Ma and monomer (F5M), dimer (Fm5MX2) and tetramer (F5MX4) of F5M were transformed into *E. coli* HMS174 (DE3) after cloning them into *Nde* I site of pGNX2 and at *Nde* I site of pT7K2.1. Fusion protein was expressed following the method in Example 6, and the expression level was quantified by scanning the results from SDS -PAGE by a densitometer and as the percent of fusion peptide in total cell proteins. In Figure 15, lanes 1-6 in pT7K2.1 represent F4Ma, F4MaX2, F4MaX4, F5M, F5MX2, and F5MX4, respectively. Lanes 1 - 4 in pGNX2 represent F4Ma, F4MaX2,

F5M and F5MX2, respectively. As can be seen from Figure 15, the expression level increased from 30 % to 40 % when the expression of tetramer was compared with that of the monomer. In the case of F5M, the expression level increased from 20 % to 25 % when the expression of tetramer was compared with that of monomer.

According to the present invention, antimicrobial peptides can be efficientely mass-produced from microorganisms more economically and can be separated and purified easily.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Park, Chong Hun
Samyang Genex Corp., #263, Yeongji-dong, Chongno-ku, Seoul 110-725,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: *E. coli* HMS174(DE3)/pGNX2	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0486BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on May 29 1998.

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korea Research Institute of Bioscience and Biotechnology
Korean Collection for Type Cultures

Address: KCTC, KRIBB
#52, Oun-dong, Yusŏng-ku,
Taejŏn 305-333,
Republic of Korea

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Bae K S.

Kyung Sook Bae, Curator
Date: June 03 1998

What is claimed is:

1. A DNA construct comprising a first sequence coding for an entire, a part of or a derivative of *purF* gene and a second sequence coding for an antimicrobial peptide.

2. A DNA construct according to Claim 1 wherein the *purF* gene is derived from a microorganism.

3. A DNA construct according to Claim 1 wherein the DNA construct is a multimeric DNA construct composed of repeatitive units of 1) a first restriction enzyme site that can generate an initiation codon Met, 2) a DNA construct, 3) a ribosome binding site (RBS), and 4) a second restriction enzyme site generating a cohesive end which can be in-frame fused to the cohesive end generated by the first restriction enzyme and thus generate the initiation codon.

4. An expression vector that is composed of a high copy number replication origin, a strong transcription promoter and a structural gene without possessing $lacI^Q$ gene, and where an expression of a foreign gene can be induced by lactose.

5. An expression vector according to Claim 4 wherein the expression product by the vector is expressed as a water-insoluble form.

6. A vector selected from the group consisting of pGNX2, pGNX3, pGNX4 and pGNX5.

7 A production method of antimicrobial peptide comprising the following steps;

constructing an expression vector containing a genetic construct comprising a first sequence coding for an entire, a part of or a derivative of *purF* gene and a second sequence coding for an antimicrobial peptide; transforming bacterial host cells with said vector; culturing the transformed cell to express a peptide as a fusion protein; and recovering the fusion protein.

8. A DNA construct comprising repeatitive units of 1) a first restriction enzyme site that can generate an initiation codon Met, 2) a DNA construct, 3) a ribosome binding site (RBS), and 4) a second restriction enzyme site generating a cohesive end which can be in-frame fused to a cohesive end generated by the first restriction enzyme and thus generate the initiation codon.

FIG. 1

APIDAECIN I

GGT AAC AAC CGT CCG GTT TAC ATC CCG CAG CCG CGT CCG CCG CAC CCG CGT ACT TGA

G N N R P V Y I P Q P R P P H P R I

EcoR I

GAATTC G

BOMBININ

GGT ATC GGT GCG CTG TCT GCG AAA GGT GCG CTG AAA GGT CTG GCG AAA GGT CTG GCG

G I G A L S A K G A L K G L A K G L A

EcoR I

GAA CAC TTC GCG AAC TGA GAATTC G

E H F A N

CPF I

GGT TTC GCG TCT TTC CTG GGT AAA GCG CTG AAA GCG CTG AAA GCG GCG CTG AAA ATC

G F A S F L G K A L K A L K A A L K I

EcoR I

GGT GCG AAC GCG CTG GGT GGT GCG CCG CAG CAG TGA GAATTC G

G A N A L G G A P Q Q

FIG.1 cont'd

DROSOCIN

GGT AAA CCG CGT CCG TAC TCT CCG CGT CCG ACC TCT CAC CCG CGT CCG ATC GCG GTT

G K P R P Y S P R P T S H P R P I A V

EcoR I

TGA GAATTC G

HNP-I

Nco I

GCATGCC ATG GCG TGC TAC TGC CGT ATC CCG GCG TGC ATC GCG GGT GAG CGT CGT TAC

A C Y C R I P A C I A G E R R Y

EcoR I

GGT ACC TGC ATC TAC CAG GGT CGT CTG TGG GCG TTC TGC TGC TGA GAATTC G

G T C I Y Q G R L W A F C C

INDOLICIDIN

EcoR I

C ATG ATC CTG CCG TGG AAA TGG CCG TGG TGG CCG TGG CGT CGT TGA GAATTC G

I L P W K W P W W P W R R

MELITTIN

GGT ACT GGT GCG GTT CTG AAA GTT CTG ACC ACC GGT CTG CCG GCG CTG ATC TCT TGG

G I G A V L K V L T T G L P A L I S W

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FIG. 1 cont'd

EcoR I

ATC AAA CGT AAA CGT CAG CAG TGA GAATTC G

I K R K R Q Q

MSI-344 (a)

Nde I

TCCGGATCCAT ATG GGT ATC GGC AAA TTC CTG AAA AAG GCT AAG AAA TTT GGT AAG GCG

M G I G K F L K K A K K F G K A

Ase I

TTC GTT AAA ATC CTG AAA AAG TAATGAAGGAGATATATTAATGC

F V K I L K K RBS

MSI-344 (b)

Sma I

GGATCCC GGG ATC GGC AAA TTC CTG AAA AAG GCT AAG AAA TTT GGT AAG GCG TTC GTT

G I G K F L K K A K K F G K A F V

Ase I

AAA ATC CTG AAA AAG TAATGAAGGAGATATATTAATGGATCC

K I L K K RBS

PGQ

GGT GTT CTG TCT AAC GTT ATC GGT ATC GGT TAC CTG AAA AAA CTG GGT ACC GGT GCG

G V L S N V I G I G Y L K K L G T G A

FIG.1 cont'd

EcoR I

CTG AAC GCG GTT CTG AAA CAG TGA GAATTC G

L N A V L K Q

TACHYPLASIN I

C ATG AAA TGG TGC TTC CGT GTT TGC TAC CGT GGT ATC TGC TAC CGT CGT TGC CGT TGA

K W C F F V C Y R G I C Y R R C R

EcoR I

GAATTC G

XPF

GGT TGG GCG TCT AAA ATC GGT CAG ACC CTG GGT AAA ATC GCG AAA GTT GGT CTG AAA

G W A S K I G Q T L G K I A K V G L K

EcoR I

GAA CTG ATC CAG CCG AAA TGA GAATTC G

E L I Q P K

BUFORIN I

GGC GCG GGA CGC GGC AAA CAA GGA GGC AAA GTG CGG GCT AAG GCC AAG ACC CGC TCA

G A G R G K Q G G K V R A K A K T R S

TCC CGG GCA GGG CTC CAG TTC CCG GTC GGC CGT GTG CAC AGG CTC CTC CGC AAG GGC

S R A G L Q F P V G R V H R L L R K

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FIG.1 cont'd

*Bam*HI

AAC TAC TAA GGATCC

G N Y

BUFORIN II

GGG ACC CGT TCC TCC CGT GCT GGT CTG CAG TTC CCG GTT GGT CGT GTT CAC CGT CTG

G T R S S R A G L Q F P V G R V H R L

*Bam*HI

CTG CGT AAA TAA TGA AGG AGA TAT ATT AAT GGATCC

L R K

BUFORIN II a

GGG CGT GCT GGT CTG CAG TTC CCG GTT GGT CGT GTT CAC CGT CTG CTG CGT AAA TAA

G R A G L Q F P V G R V H R L L R K

*Bam*HI

TGA AGG AGA TAT ATT AAT GGATCC

BUFORIN II b

GGG CGT GCT GGT CTG CAG TTC CCG GTT GGT CGC CTG CTG CGC CGT CTG CTG CGT CGC

G R A G L Q F P V G R L L R R L L R R

*Bam*HI

CTG CTG CGC TAA TGA AGG AGA TAT ATT AAT GGATCC

L L R

FIG.2

F

NdeI

1 CATATGTGCGGTATTGTCGGTATCGCCGGTGTTATGCCGGTTAACCAGTC

M C G I V G I A G V M P V N Q S

51 GATTTATGATGCCTTAACGGTGCTTCAGCATCGCGGTCAGGATGCCGCCG

I Y D A L T V L Q H R G Q D A A

101 GCATCATCACCATAGATGCCAATAACTGCTTCCGTTTGCGTAAAGCGAAC

G I I T I D A N N C F R L R K A N

NdeI

151 GGGCTGGTGAGCGATGTATTTGAAGCTCGCCATATG

G L V S D V F E A R H M

F'

NdeI

1 CATATGTGCGGTATTGTCGGTATCGCCGGTGTTATGCCGGTTAACCAGTC

M C G I V G I A G V M P V N Q S

51 GATTTATGATGCCTTAACGGTGCTTCAGCATCGCGGTCAGGATGCCGCCG

I Y D A L T V L Q H R G Q D A A

101 GCATCATCACCATAGATGCCAATAACTGCTTCCGTTTGCGTAAAGCGAAC

G I I T I D A N N C F R L R K A N

FIG.2 cont'd

SspI

151 GCGCTGGTGAGCGATGTATTTGAAGCTAATATT

A L V S D V F E A N

F3 (HA)

NdeI

1 CATATGTGCGGTATTGTCGGTATCGCCGGTGTTATGCCGGTTAACCAGTC

M C G I V G I A G V M P V N Q S

51 GATTTATGATGCCTTAACGGTGCTTCAGCATCCGCGGTCAGGATGCCGCCG

I Y D A L T V L Q H R G Q D A A

101 GCATCATCACCATAGATGCCAATAACTGCTTCCGTTTGCGTAAAGCGAAC

G I I T I D A N N C F R L R K A N

SspI

151 GCGCTGGTGAGCGATGTATTTGAAGCTGCGCATGCGAATATT

A L V S D V F E A A H A N

F3(CB)

AS SAME AS 1-100 F3(HA)

BspLU11 I

151 GCGCTGGTGAGCGATGTATTTGAAGCTCGCCACATGTGGATCCCG

A L V S D V F E A R H M

FIG.2 cont'd

F4(HA)

NdeI

1 CATATGTGCGGTATTGTCGGTATCGCCGGTGTTATGCCGGTTAACCAGTC

M C G I V G I A G V M P V N Q S

51 GATTTATGATGCCTTAACGGTGCTTCAGCATCGCGGTCAGGATGCCGCCG

I Y D A L T V L Q H R G Q D A A

101 GCATCATCACCATAGATGCCAATAACTGCTTCCGTTTGCGTAAAGCGAAC

G I I T I D A N N C F R L R K A N

NdeI

151 GGGCTGGTGAGCGATGTATTTGAAGCTCGCCATATGCAGCGTTTGCAGGG

G L V S D V F E A R H M Q R L Q G

201 CAATATGGGCATTGGTCATGTGCGTTACCCCACGGCTGGCAGCTCCAGCG

N M G I G H V R Y P T A G S S S

251 CCTCTGAAGCGCAGCCGTTTTACGTTAACTCCCCGTATGGCATTACGCTT

A S E A Q P F Y V N S P Y G I T L

301 GCCCACATCGGCAATCTGACCAACGCTCACGAGTTGCGTAAAAAACTGTT

A H I G N L T N A H E L R K K L F

351 TGAAGAAAAACGCCGCCACATCAACACCACTTCCGACTCGGAAATTCTGC

E E K R R H I N T T S D S E I L

FIG.2 cont'd

401 TTAATATCTTCGCCAGCGAGCTGGACAACTTCCGCCACTACCCGCTGGAA

L N I F A S E L D N F R H Y P L E

SspI

451 GCCGACAATATT

A D N

F4a(HA)

AS SAME AS 1-150 F4(HA)

151 GCGCTGGTGAGCGATGTATTTGAAGCTCGCCATATGCAGCGTTTGCAGGG

A L V S D V F E A R H M Q R L Q G

AS SAME AS 201-462 F4(HA)

F4a(CB)

AS SAME AS 1-450 F4M(HA)

BspLU11 I

451 GCCGACATGTGG

A D M

F5

NdeI

1 CATATGCAGCGTTTGCAGGGCAATATGGGCATTGGTCATGTGCGTTACCC

M Q R L Q G N M G I G H V R Y P

FIG.2 cont'd

51 CACGGCTGGCAGCTCCAGCGCCTCTGAAGCGCAGCCGTTTTACGTTAACT

T A G S S S A S E A Q P F Y V N

101 CCCCGTATGGCATTACGCTTGCCCACATCGGCAATCTGACCAACGCTCAC

S P Y G I T L A H I G N L T N A H

151 GAGTTGCGTAAAAAACTGTTTGAAGAAAAACGCCGCCACATCAACACCAC

E L R K K L F E E K R R H I N T T

201 TTCCGACTCGGAAATTCTGCTTAATATCTTCGCCAGCGAGCTGGACAACT

S D S E I L L N I F A S E L D N

SspI

251 TCCGCCACTACCCGCTGGAAGCCGACAATATT

F R H Y P L E A D N

BF

NdeI

1 CATATGCTTGCTGAAATCAAAGGCTTAAATGAAGAATGCGGCGTTTTTGG

M L A E I K G L N E E C G V F G

51 GATTTGGGGACATGAAGAAGCCCCGCAAATCACGTATTACGGTCTCCACA

I W G H E E A P Q I T Y Y G L H

SspI

101 GCCTTCAGCACCGAGGACAGGAGGGTGCTGGCAATATT

S L Q H R G Q E G A G N

FIG. 3

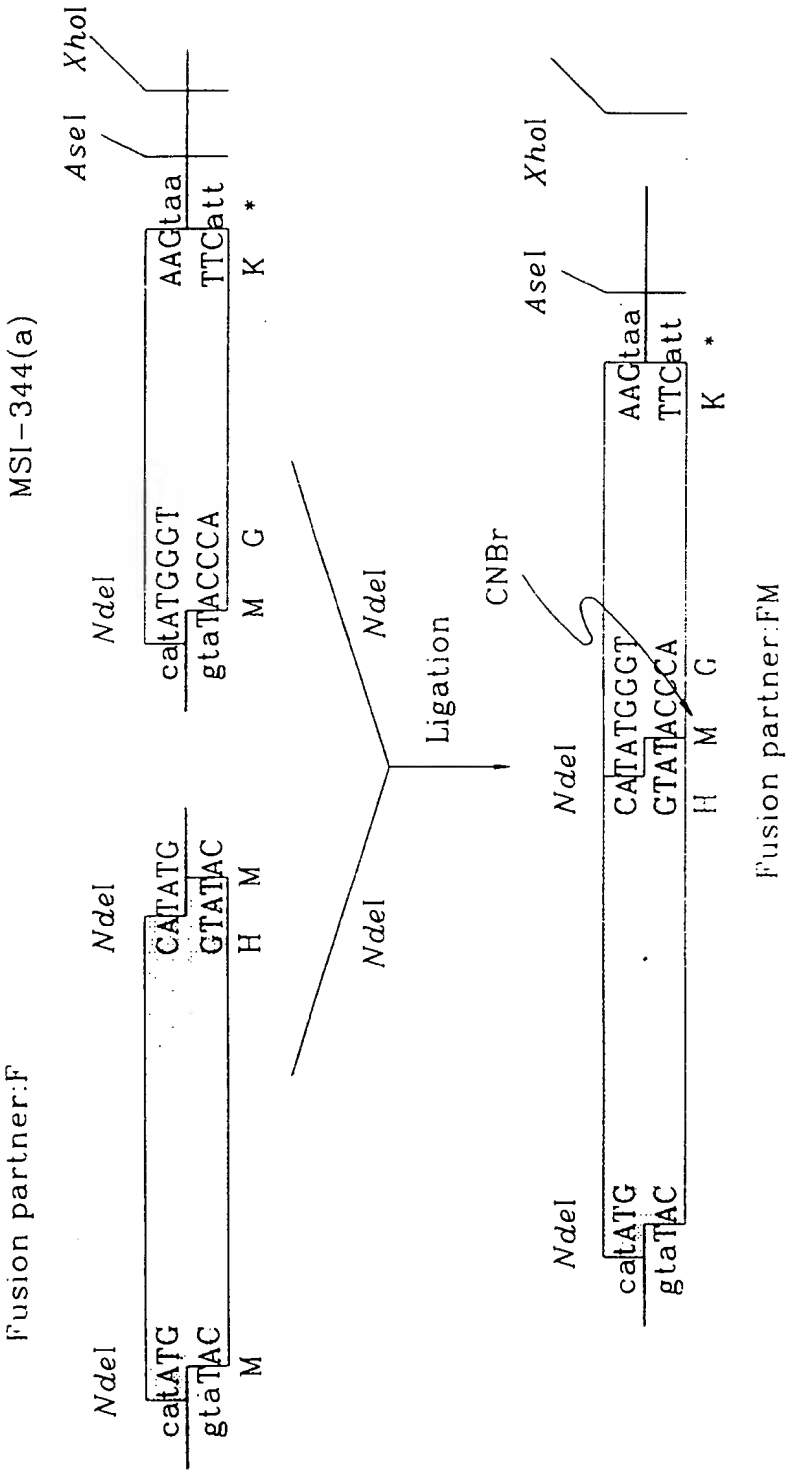

MSI-344(a)
NdeI
AseI
XhoI
catATGGGT
gtaTACCCA
AAGtaa
TTCatt
M G
K *
Fusion partner:F
NdeI
catATG
gtaTAC
CATATG
GTATAC
M
H M
Ligation
CNBr
Fusion partner:FM
CATATGGGT
GTATACCCA
H M G

FIG. 3 cont'd

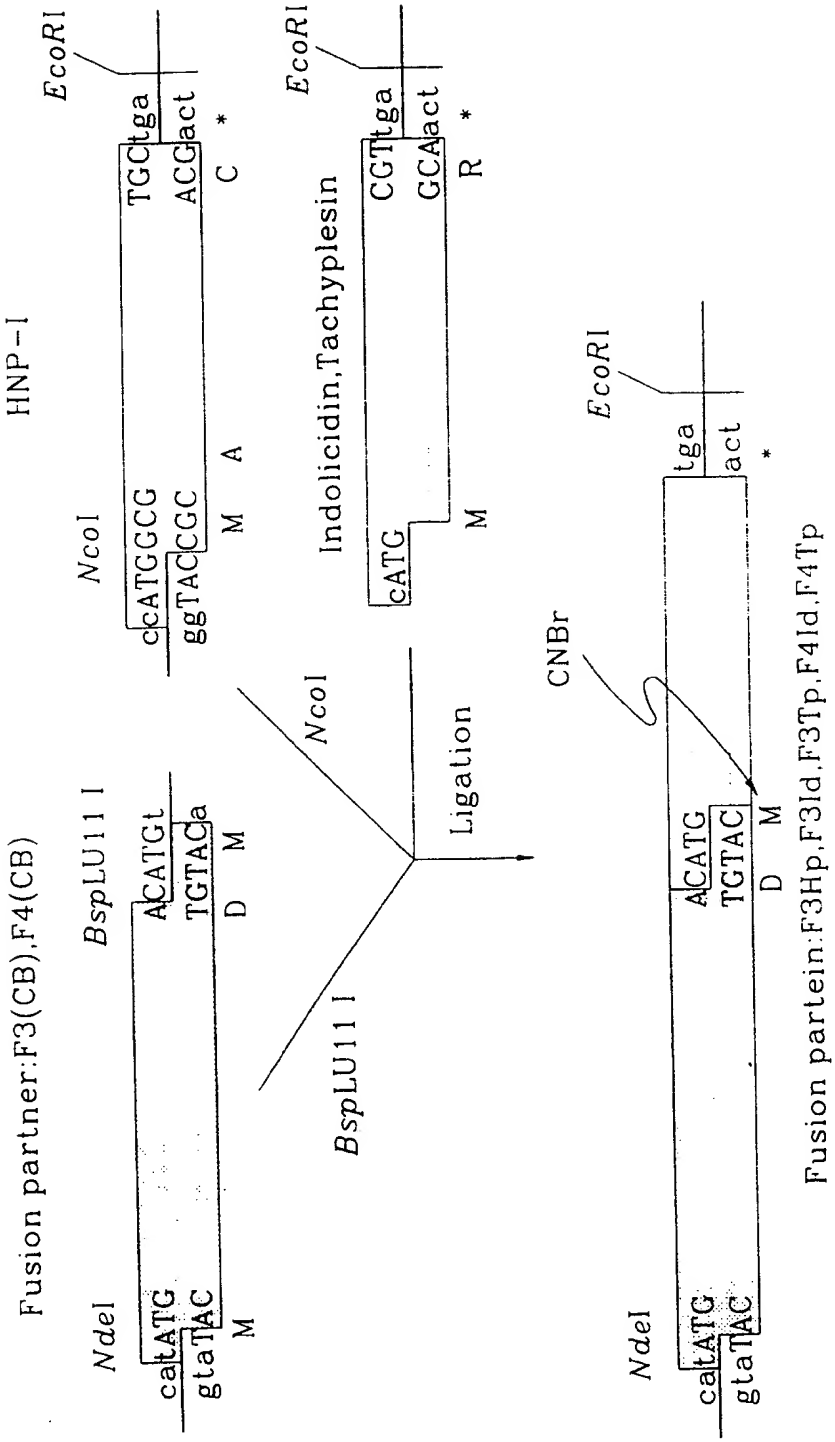

Fusion partner:F3(CB),F4(CB)
HNP-1
NdeI
catATG
gtaTAC
M
BspLU11 I
ACATGt
TGTACa
D M
NcoI
cATGGCG
ggTACCGC
M A
EcoRI
TGCtga
ACGact
C *
NcoI
BspLU11 I
Ligation
Indolicidin,Tachyplesin
cATG
M
CGTtga
GCAact
R *
EcoRI
CNBr
NdeI
catATG
gtaTAC
ACATG
TGTAC
D M
tga
act
*
EcoRI
Fusion partein:F3Hp,F3Id,F3Tp,F4Id,F4Tp

FIG. 4

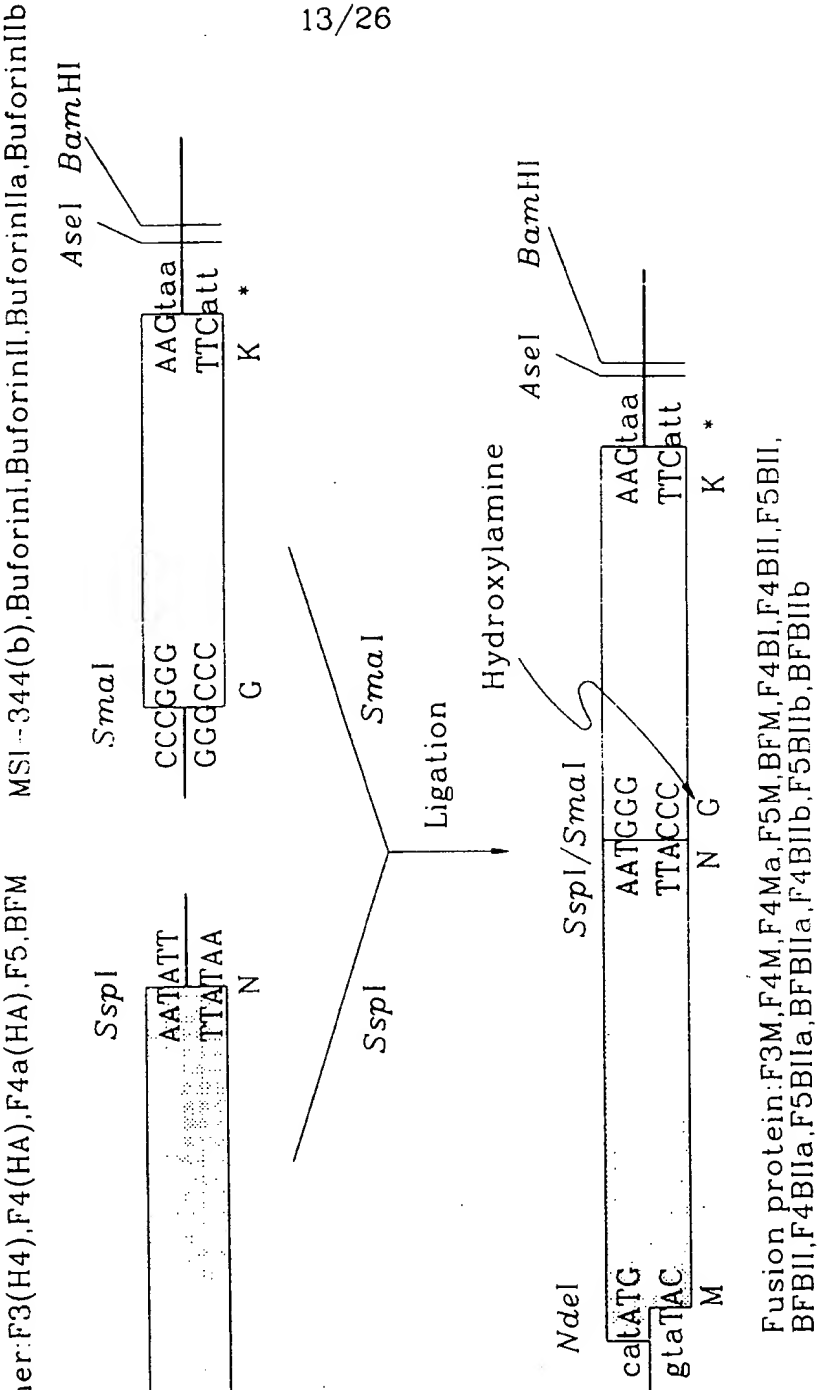

Fusion partner:F3(H4),F4(HA),F4a(HA),F5,BFM
MSI-344(b),BuforinI,BuforinII,BuforinIIa,BuforinIIb
NdeI
SspI
SmaI
AseI
BamHI
catATG
gtaTAC
AATATT
TTATAA
CCCGGG
GGGCCC
AAGtaa
TTCatt
M
N
G
K
*
Ligation
Hydroxylamine
SspI/SmaI
AATGGG
TTACCC
N
G
Fusion protein:F3M,F4M,F4Ma,F5M,BFM,F4BI,F4BII,F5BII,
BFBII,F4BIIa,F5BIIa,BFBIIa,F4BIIb,F5BIIb,BFBIIb

FIG. 4 contd

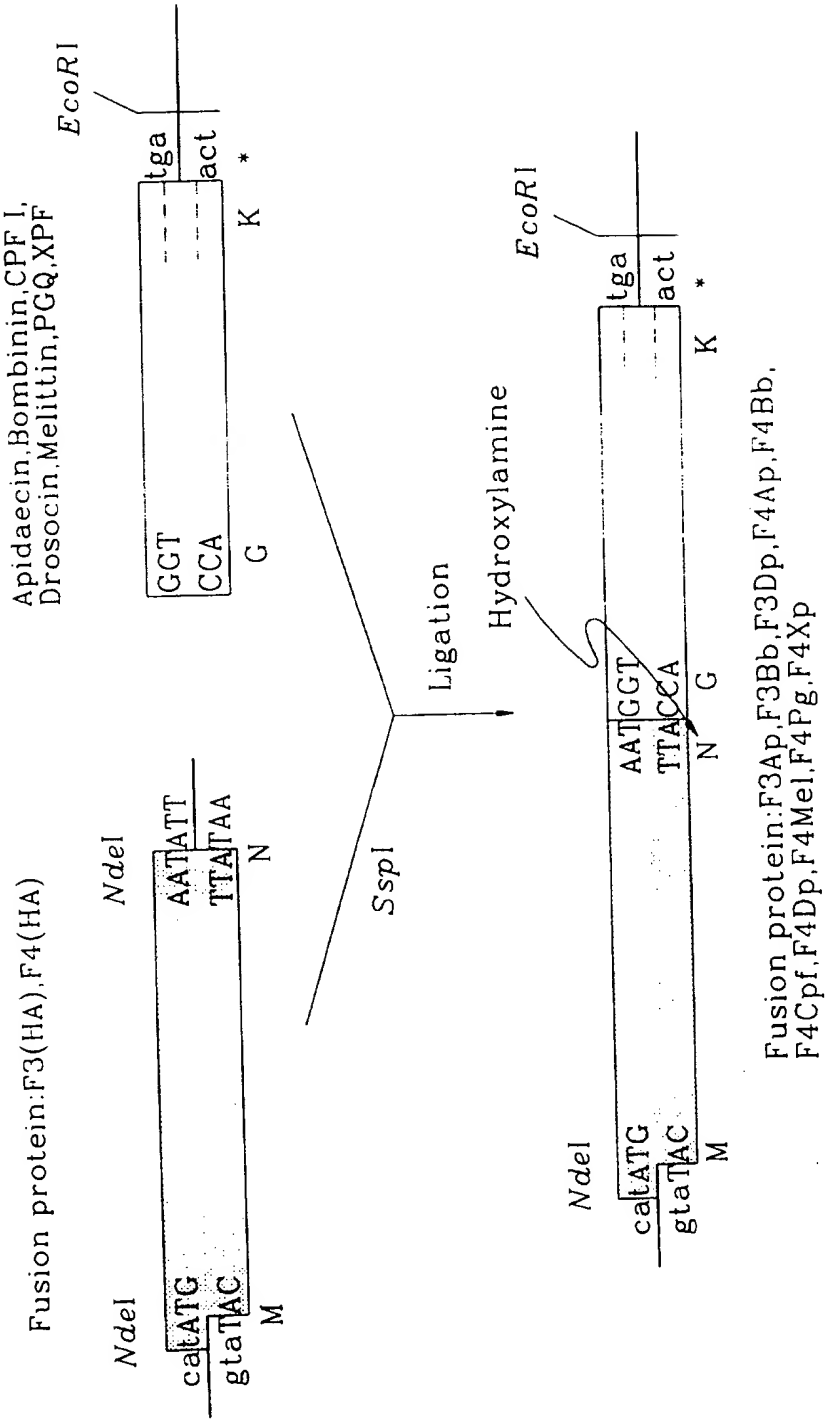

Fusion protein:F3(HA),F4(HA)
NdeI
catATG
gtaTAC
M
AATATT
TTATAA
N
SspI
Ligation
Hydroxylamine
Apidaecin,Bombinin,CPF I,
Drosocin,Melittin,PGQ,XPF
EcoRI
GGT
CCA
G
tga
act
K
*
NdeI
catATG
gtaTAC
M
AATGGT
TTACCA
N
G
EcoRI
tga
act
K
*
Fusion protein:F3Ap,F3Bb,F3Dp,F4Ap,F4Bb,
F4Cpf,F4Dp,F4Mel,F4Pg,F4Xp

FIG. 5

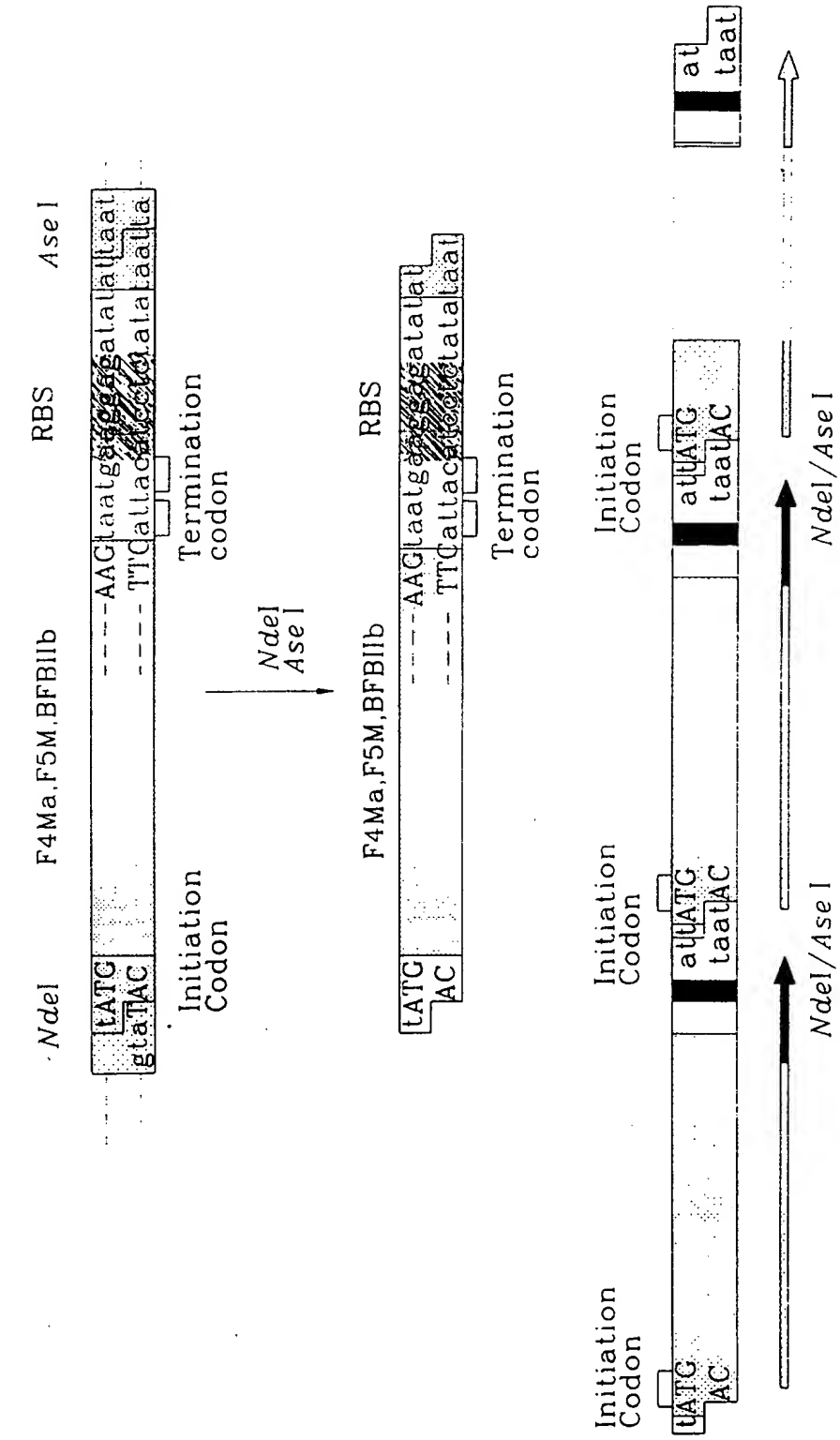

NdeI
F4Ma,F5M,BFBIIb
RBS
AseI
Initiation Codon
Termination codon
NdeI
AseI
F4Ma,F5M,BFBIIb
RBS
Initiation Codon
Termination codon
Initiation Codon
Initiation Codon
Initiation Codon
NdeI/AseI
NdeI/AseI

FIG 6

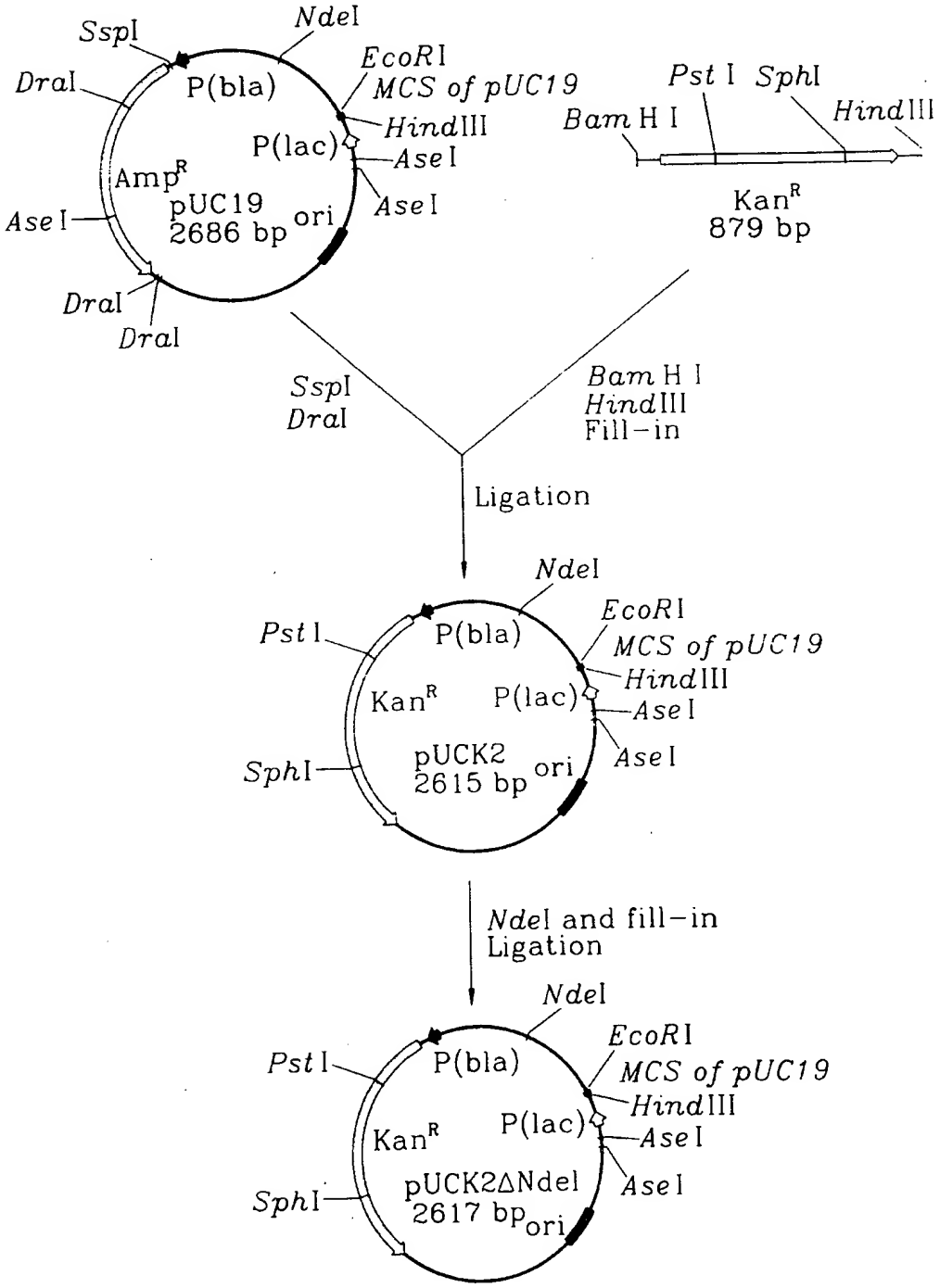

SspI
NdeI
EcoRI
MCS of pUC19
HindIII
AseI
AseI
DraI
P(bla)
P(lac)
AmpR
pUC19
2686 bp
ori
AseI
DraI
DraI
Pst I
SphI
Bam H I
HindIII
KanR
879 bp
SspI
DraI
Bam H I
HindIII
Fill-in
Ligation
NdeI
EcoRI
MCS of pUC19
HindIII
AseI
AseI
PstI
P(bla)
KanR
P(lac)
pUCK2
2615 bp
ori
SphI
NdeI and fill-in
Ligation
NdeI
EcoRI
MCS of pUC19
HindIII
AseI
AseI
PstI
P(bla)
KanR
P(lac)
pUCK2ΔNdeI
2617 bp
ori
SphI

FIG. 6 cont'd

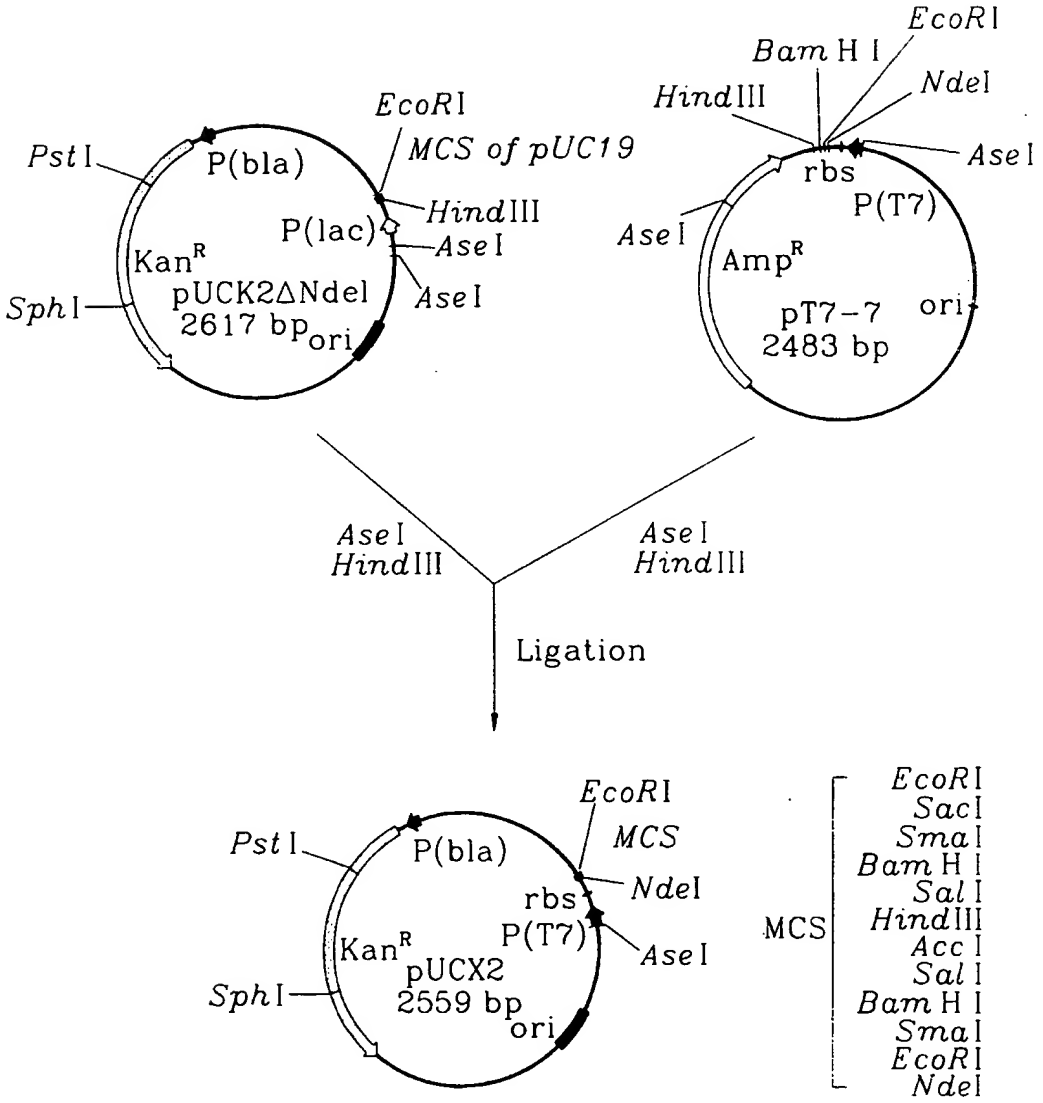

EcoRI
Bam H I
HindIII
NdeI
AseI
rbs
P(T7)
AseI
Amp^R
pT7-7
2483 bp
ori
EcoRI
MCS of pUC19
PstI
P(bla)
HindIII
P(lac)
AseI
Kan^R
pUCK2ΔNdeI
2617 bp
ori
SphI
AseI
HindIII
AseI
HindIII
Ligation
EcoRI
MCS
PstI
P(bla)
NdeI
rbs
P(T7)
AseI
Kan^R
pUCX2
2559 bp
ori
SphI
MCS
EcoRI
SacI
SmaI
Bam H I
SalI
HindIII
AccI
SalI
Bam H I
SmaI
EcoRI
NdeI

FIG. 7

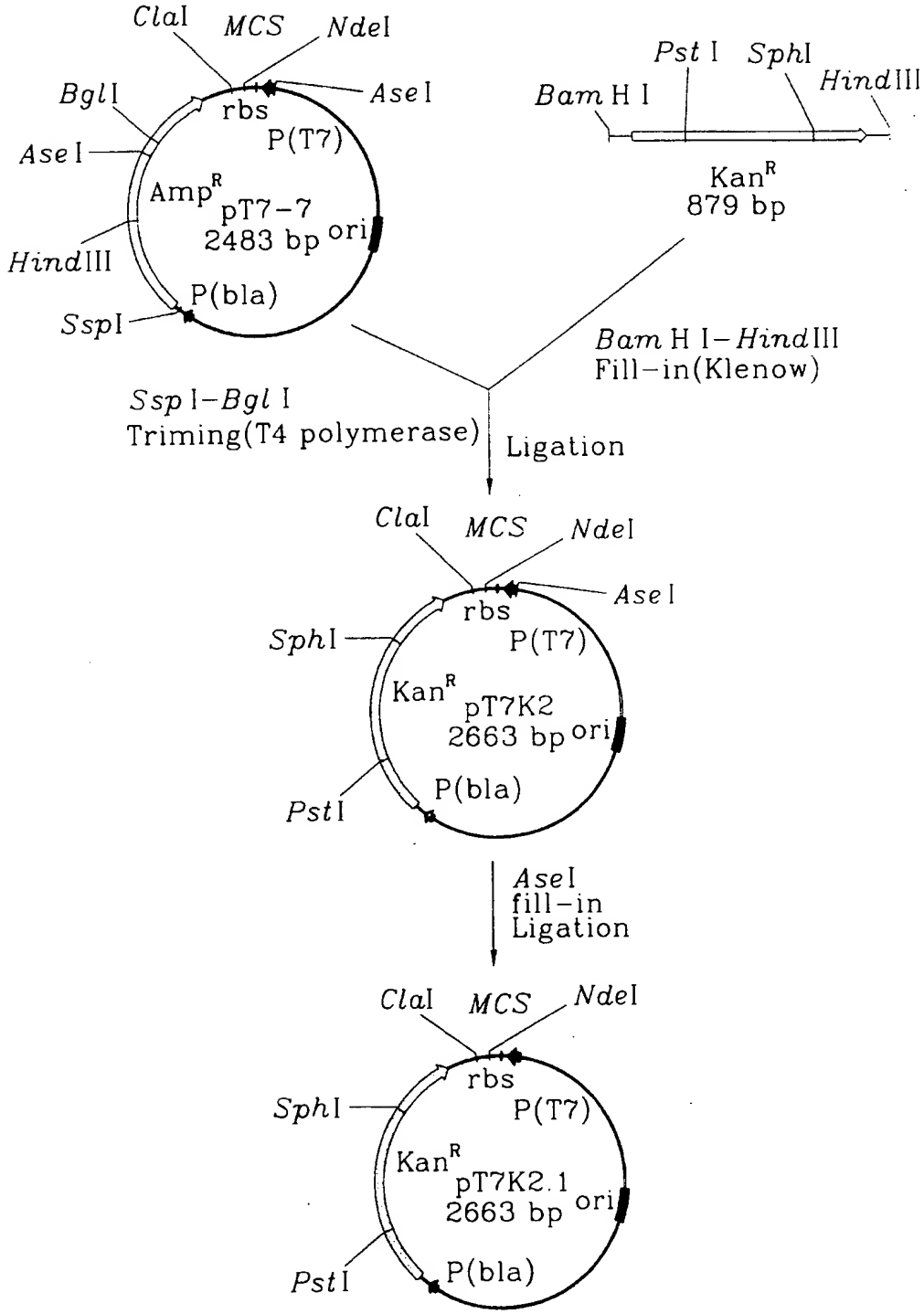

ClaI
MCS
NdeI
AseI
BglI
rbs
P(T7)
AseI
Amp^R
pT7-7
2483 bp
ori
HindIII
P(bla)
SspI
Pst I
SphI
HindIII
BamH I
Kan^R
879 bp
BamH I-HindIII
Fill-in(Klenow)
SspI-BglI
Triming(T4 polymerase)
Ligation
ClaI
MCS
NdeI
AseI
rbs
SphI
P(T7)
Kan^R
pT7K2
2663 bp
ori
P(bla)
PstI
AseI
fill-in
Ligation
ClaI
MCS
NdeI
rbs
SphI
P(T7)
Kan^R
pT7K2.1
2663 bp
ori
PstI
P(bla)

FIG. 8

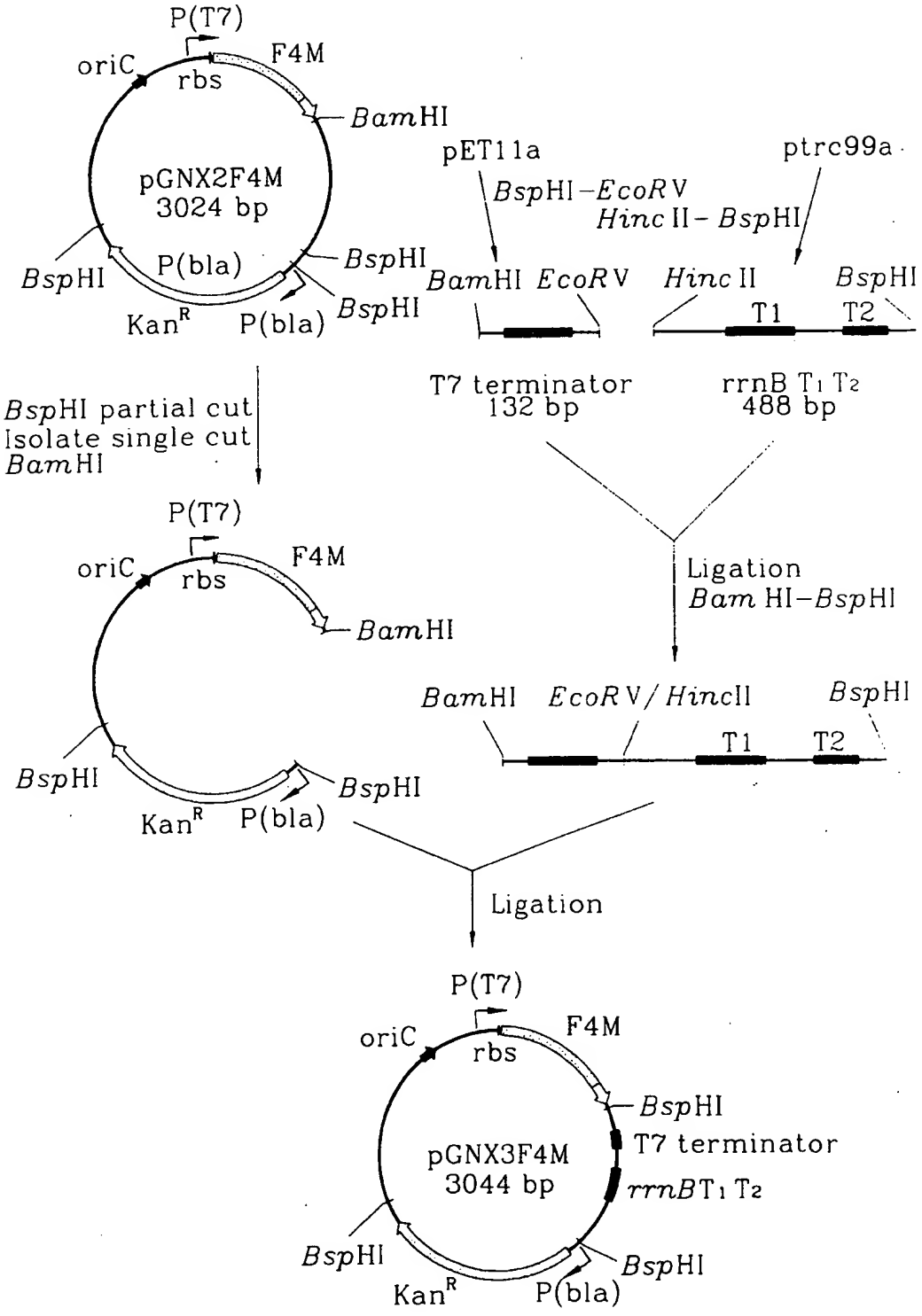

P(T7)
oriC
rbs
F4M
BamHI
pGNX2F4M
3024 bp
BspHI
P(bla)
BspHI
BspHI
Kan^R
P(bla)
BspHI
pET11a
BspHI-EcoRV
ptrc99a
Hinc II- BspHI
BamHI EcoRV
Hinc II
BspHI
T1
T2
T7 terminator
132 bp
rrnB T1 T2
488 bp
BspHI partial cut
Isolate single cut
BamHI
Ligation
Bam HI-BspHI
BamHI EcoRV/HincII
BspHI
T1
T2
Ligation
P(T7)
oriC
rbs
F4M
BamHI
BspHI
BspHI
Kan^R
P(bla)
P(T7)
oriC
rbs
F4M
BspHI
T7 terminator
pGNX3F4M
3044 bp
rrnBT1 T2
BspHI
BspHI
Kan^R
P(bla)

FIG. 9

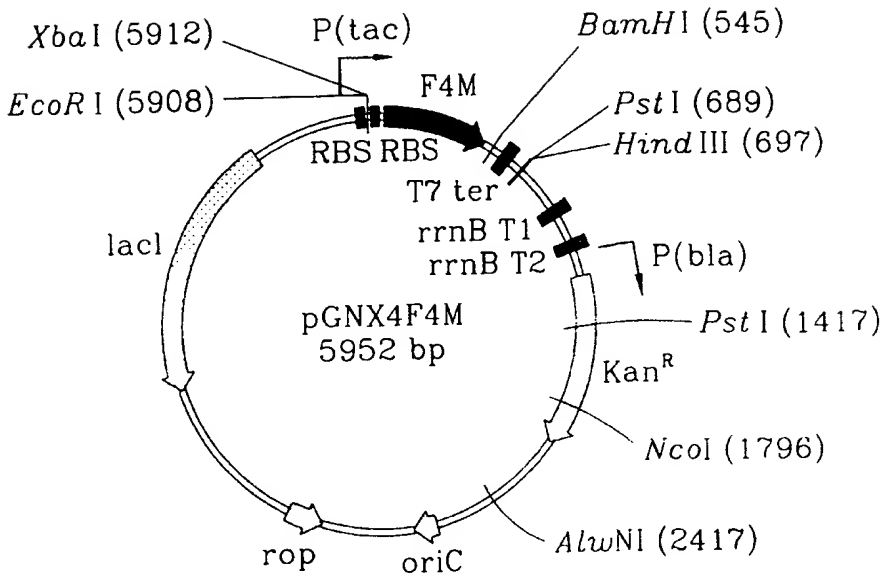

XbaI (5912)
P(tac)
BamHI (545)
EcoR I (5908)
F4M
PstI (689)
RBS RBS
HindIII (697)
T7 ter
rrnB T1
rrnB T2
P(bla)
lacI
pGNX4F4M
5952 bp
PstI (1417)
KanR
NcoI (1796)
AlwNI (2417)
rop
oriC

FIG. 10

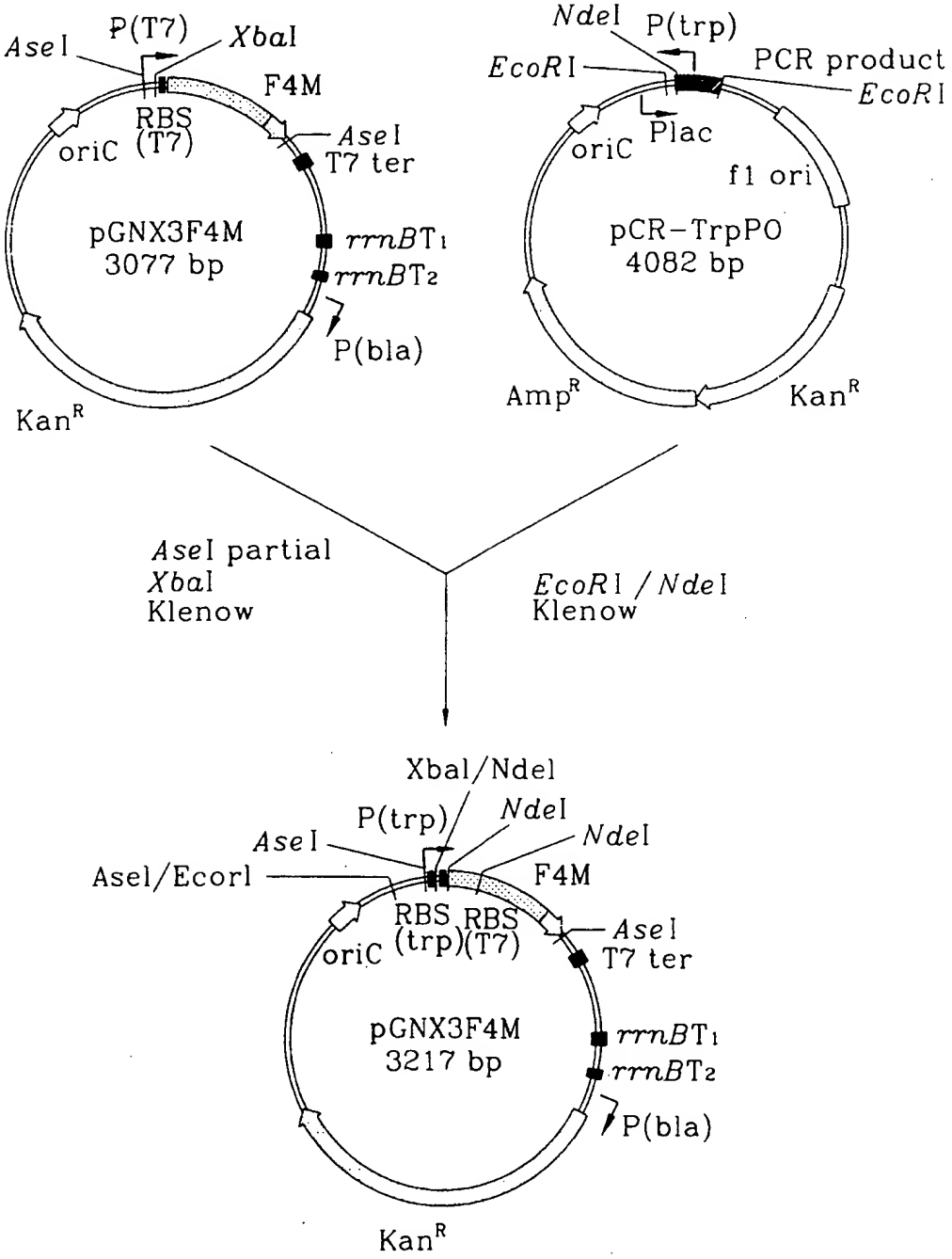

AseI
P(T7)
XbaI
F4M
RBS
(T7)
oriC
AseI
T7 ter
pGNX3F4M
3077 bp
rrnBT1
rrnBT2
P(bla)
Kan^R
NdeI
P(trp)
EcoRI
PCR product
EcoRI
Plac
oriC
f1 ori
pCR-TrpPO
4082 bp
Amp^R
Kan^R
AseI partial
XbaI
Klenow
EcoRI / NdeI
Klenow
XbaI/NdeI
P(trp)
NdeI
NdeI
AseI
AseI/EcoRI
F4M
RBS
(trp)
RBS
(T7)
oriC
AseI
T7 ter
pGNX3F4M
3217 bp
rrnBT1
rrnBT2
P(bla)
Kan^R

FIG. 11

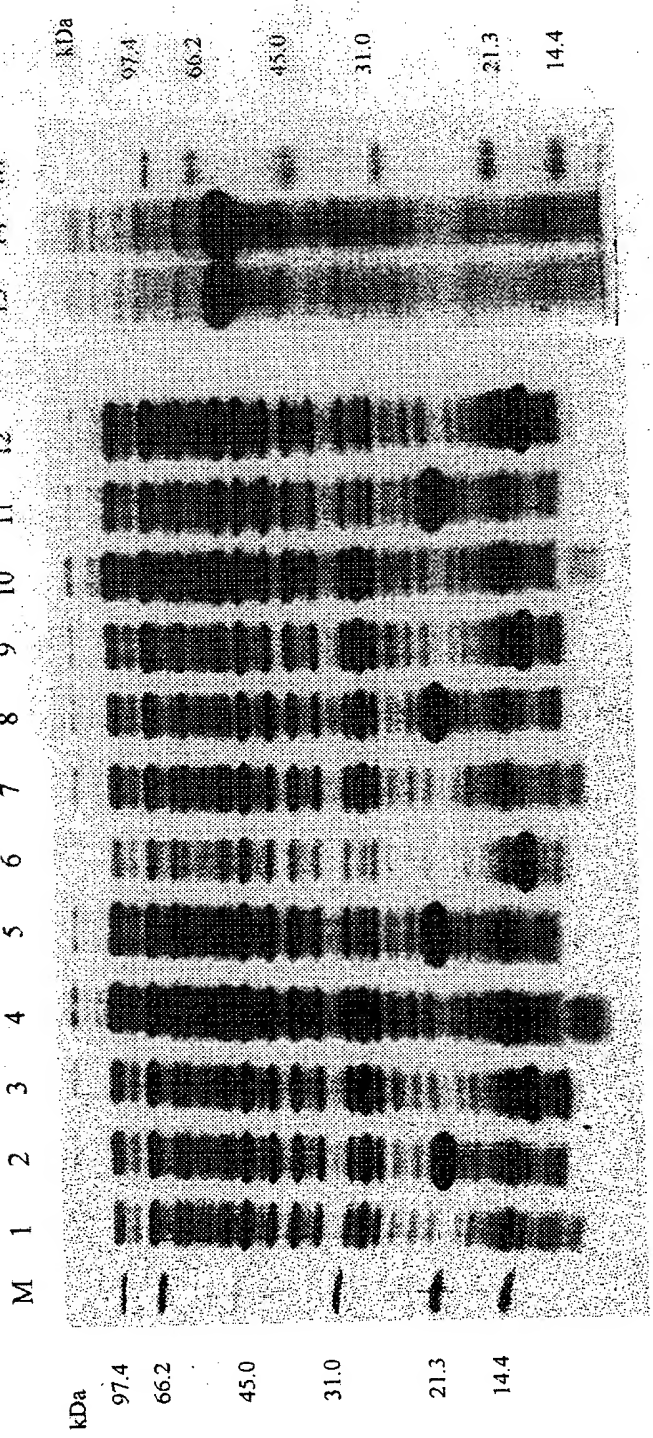

kDa
97.4
66.2
45.0
31.0
21.3
14.4
M
1
2
3
4
5
6
7
8
9
10
11
12
13
14
M
kDa
97.4
66.2
45.0
31.0
21.3
14.4

FIG. 12

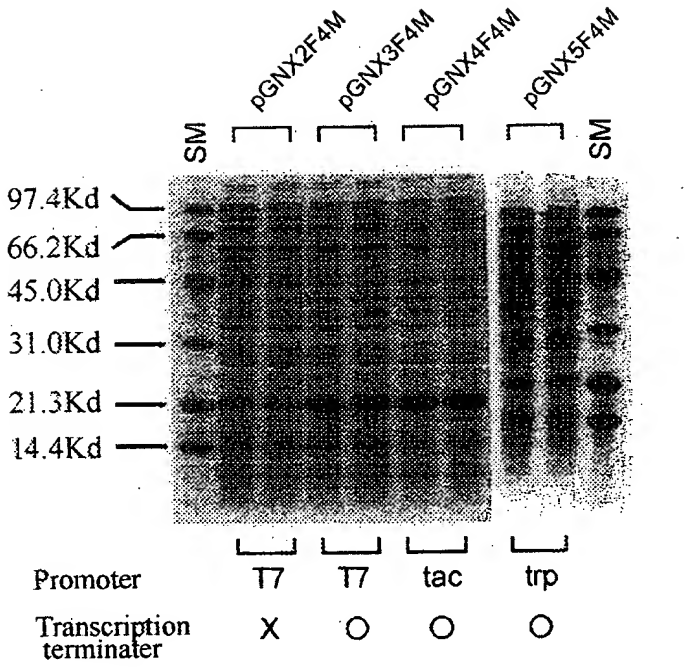
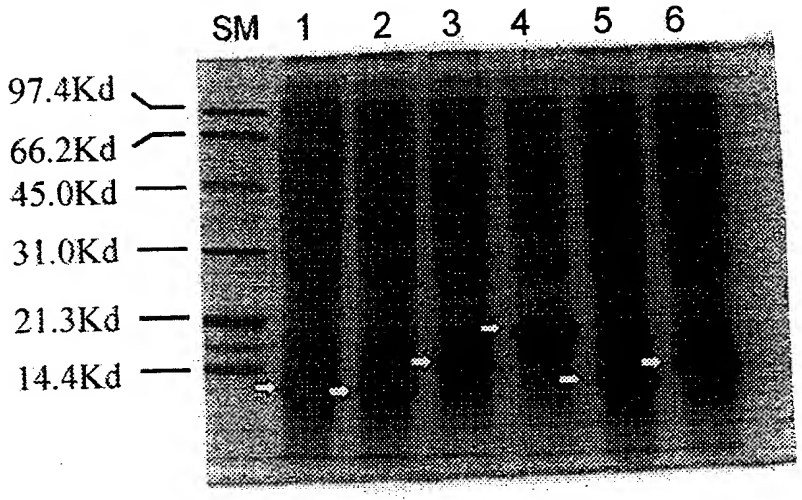
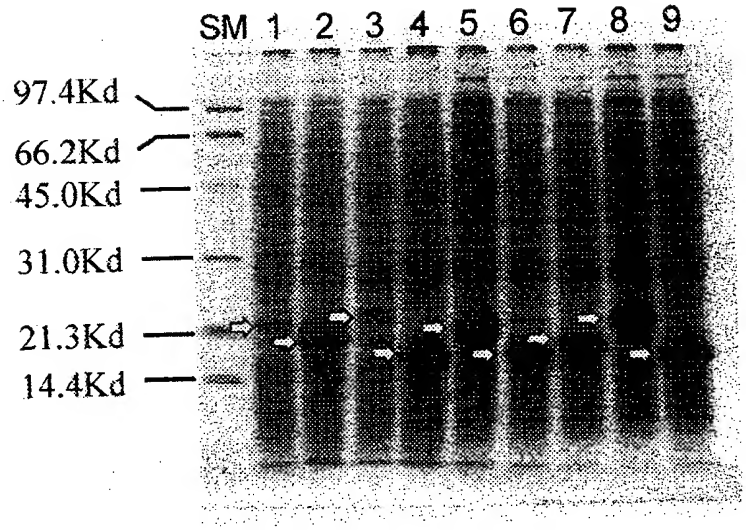

SM
pGNX2F4M
pGNX3F4M
pGNX4F4M
pGNX5F4M
SM
97.4Kd
66.2Kd
45.0Kd
31.0Kd
21.3Kd
14.4Kd
Promoter
T7
T7
tac
trp
Transcription terminater
X
O
O
O

FIG. 13


SM 1 2 3 4 5 6
97.4Kd
66.2Kd
45.0Kd
31.0Kd
21.3Kd
14.4Kd

(A)


SM 1 2 3 4 5 6 7 8 9
97.4Kd
66.2Kd
45.0Kd
31.0Kd
21.3Kd
14.4Kd

(B)

FIG. 14

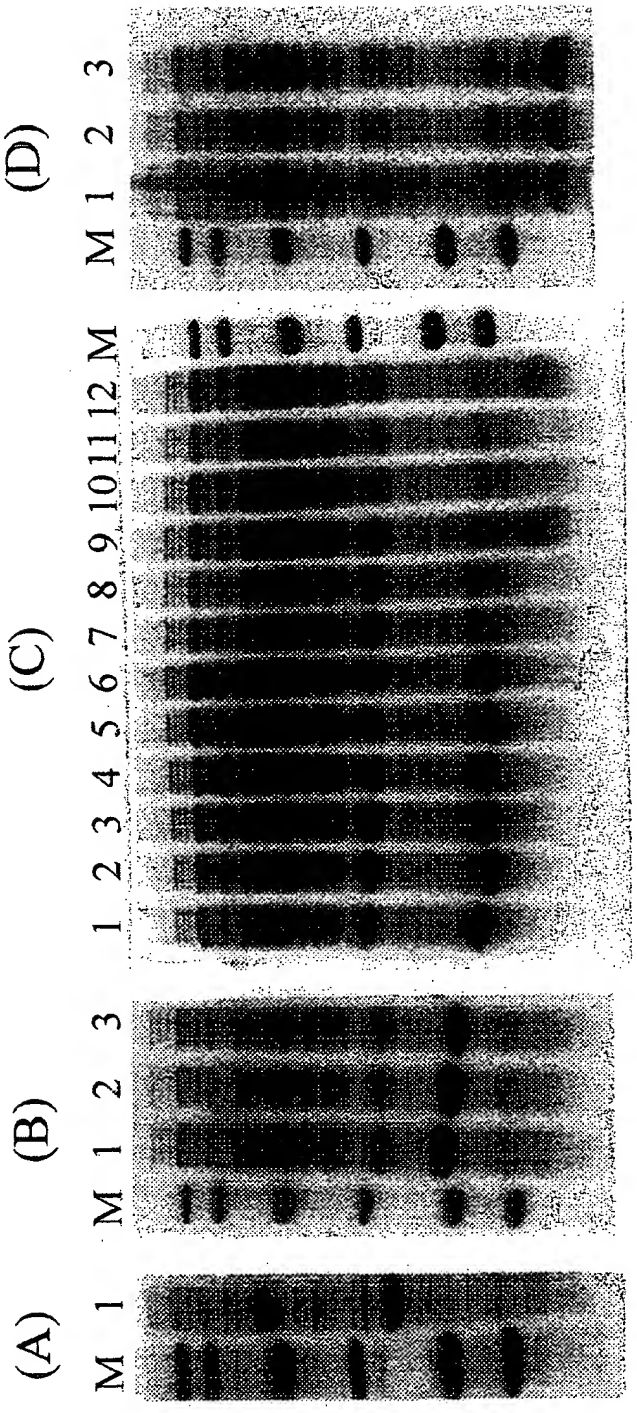
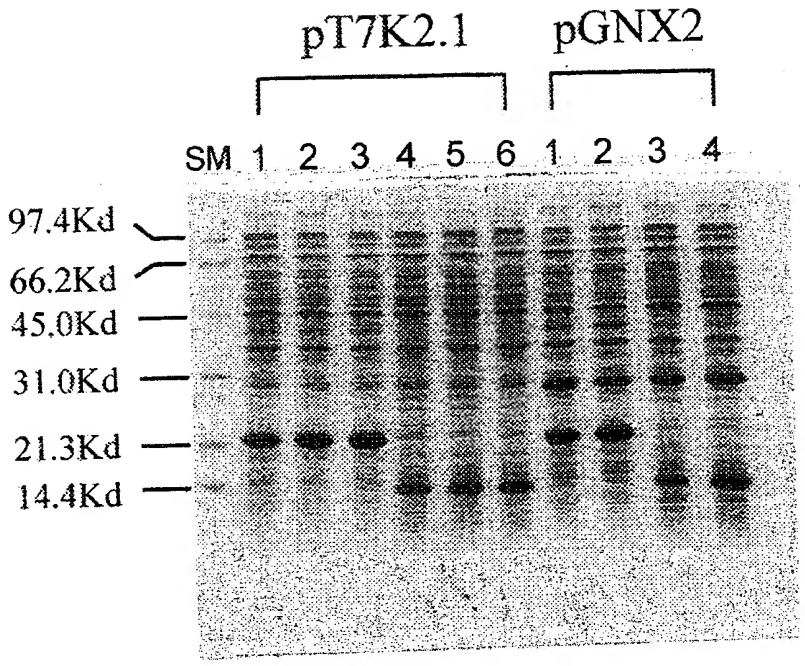


FIG. 15


pT7K2.1
pGNX2
SM 1 2 3 4 5 6 1 2 3 4
97.4Kd
66.2Kd
45.0Kd
31.0Kd
21.3Kd
14.4Kd

SEQUENCE LISTING

<110> PARK, Chong-Hun; Samyang Genex Corporation

<120> Mass production method of antimicrobial peptide and DNA construct and expr
ession system thereof

<130> PA/SYG/99243

<150> KR 1998-22117
<151> 1998-06-09

<150> KR 1999-17920
<151> 1999-05-14

<160> 9

<170> KOPATIN 1.0

<210> 1
<211> 186
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 1
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gggctggtga gcgatgtatt tgaagctcgc 180

catatg 186

<210> 2
<211> 183
<212> DNA
<213> Artificial Sequence

SEQUENCE LISTING

<220>
<223> part of E.coli purF

<400> 2
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gcgctggtga gcgatgtatt tgaagctaat 180

att 183

<210> 3
<211> 192
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 3
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gcgctggtga gcgatgtatt tgaagctgcg 180

catgcgaata tt 192

<210> 4
<211> 145
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 4

SEQUENCE LISTING

catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcgctggtga gcgatgtatt 120

tgaagctcgc cacatgtgga tcccg 145

<210> 5
<211> 462
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 5
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gggctggtga gcgatgtatt tgaagctcgc 180

catatgcagc gtttgcaggg caatatgggc attggtcatg tgcgttaccc cacggctggc 240

agctccagcg cctctgaagc gcagccgttt tacgttaact ccccgtatgg cattacgctt 300

gcccacatcg gcaatctgac caacgctcac gagttgcgta aaaaactgtt tgaagaaaaa 360

cgccgccaca tcaacaccac ttccgactcg gaaattctgc ttaatatctt cgccagcgag 420

ctggacaact tccgccacta cccgctggaa gccgacaata tt 462

<210> 6
<211> 462
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

SEQUENCE LISTING

<400> 6
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gcgctggtga gcgatgtatt tgaagctcgc 180

catatgcagc gtttgcaggg caatatgggc attggtcatg tgcgttaccc cacggctggc 240

agctccagcg cctctgaagc gcagccgttt tacgttaact ccccgtatgg cattacgctt 300

gcccacatcg gcaatctgac caacgctcac gagttgcgta aaaaactgtt tgaagaaaaa 360

cgccgccaca tcaacaccac ttccgactcg gaaattctgc ttaatatctt cgccagcgag 420

ctggacaact tccgccacta cccgctggaa gccgacaata tt 462

<210> 7
<211> 462
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 7
catatgtgcg gtattgtcgg tatcgccggt gttatgccgg ttaaccagtc gatttatgat 60

gccttaacgg tgcttcagca tcgcggtcag gatgccgccg gcatcatcac catagatgcc 120

aataactgct tccgtttgcg taaagcgaac gcgctggtga gcgatgtatt tgaagctcgc 180

catatgcagc gtttgcaggg caatatgggc attggtcatg tgcgttaccc cacggctggc 240

agctccagcg cctctgaagc gcagccgttt tacgttaact ccccgtatgg cattacgctt 300

gcccacatcg gcaatctgac caacgctcac gagttgcgta aaaaactgtt tgaagaaaaa 360

SEQUENCE LISTING

cgccgccaca tcaacaccac ttccgactcg gaaattctgc ttaatatctt cgccagcgag 420

ctggacaact tccgccacta cccgctggaa gccgacatgt gg 462

<210> 8
<211> 282
<212> DNA
<213> Artificial Sequence

<220>
<223> part of E.coli purF

<400> 8
catatgcagc gtttgcaggg caatatgggc attggtcatg tgcgttaccc cacggctggc 60

agctccagcg cctctgaagc gcagccgttt tacgttaact ccccgtatgg cattacgctt 120

gcccacatcg gcaatctgac caacgctcac gagttgcgta aaaaactgtt tgaagaaaaa 180

cgccgccaca tcaacaccac ttccgactcg gaaattctgc ttaatatctt cgccagcgag 240

ctggacaact tccgccacta cccgctggaa gccgacaata tt 282

<210> 9
<211> 138
<212> DNA
<213> Bacillus subtilis

<400> 9
catatgcttg ctgaaatcaa aggcttaaat gaagaatgcg gcgtttttgg gatttgggga 60

catgaagaag ccccgcaaat cacgtattac ggtctccaca gccttcagca ccgaggacag 120

gagggtgctg gcaatatt 138

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00282

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/62; C 07 K 14/435, C 12 P 21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N; C 07 K; C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Internet Grateful Medline, U.S.Library of Medicine, online, CAS Database, Questel. Orbit. Imaginations, Paris (FR).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JH Lee et al. "Acidic Peptide-mediated Expression of the Antimicrobial Peptide Buforin II as Tandem Repeats in Escherichia Coli", Protein Expr Purif, Feb.1998, abstract [online][retrieved on 08.07.99] Retrieved from the Internet: Medline, Internet Grateful Medline, U.S.National Library of Medicine, Bethesda, MD USA, <http://igm.nlm.nih.gov/cgi-bin/doler?account=++&password=++& datafile =medline>, abstract.	1-3,7
A.P	L.Zhang et al. "Determinants of Recombinant Production of Antimicrobial Cationic Peptides and Creation of Peptide Variants in Bacteria", Biochem Biophys Res Commun, 29 June 1998, abstract [online][retrieved on 08.0.7.99] Retrieved from the Internet: Medline, Internet Grateful Medline, U.S.National Library of Medicine, Bethesda, MD USA, <http://igm.nlm.nih.gov/cgi-bin/doler?account =++&password=++& datafile =medline>, abstract.	1-3,7

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 1999 (10.08.99)	03 September 1999 (03.09.99)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Authorized officer Weniger Telephone No. 1/53424/458

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00282

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M Okamoto et al."Enhanced Expression of an Antimicrobial Peptide Sarcotoxin IA by GUS Fusion in Transgenic Tobacco Plants", Plant Cell Physiol, January 1998, abstract [online][retrieved on 08.07.99] Retrieved form the Internet: Medline, Internet Grateful Medline, U.S.National Library of Medicine, Bethesda, MD USA, <http://igm.nlm.nih.gov/cgi-bin/doler?account=++& password =++&datafile =medline>, abstract.	1-3,7
A	C Haught et al."Recombinant Production and Purification of Novel Antisense Antimicrobial Peptide in Escherichia Coli.", Biotechno Bioeng, January 1998, abstract [online][retrieved on 08.07.99] Retrieved from the Internet: Medline, Internet Grateful Medline, U.S.National Library of Medicine, Bethesda, MD USA, <http://igm.nlm.nih.gov/cgi-bin/doler?account=++&password=++& datafile=medline>, abstract.	1-3,7

INTERNATIONAL SEARCH REPORT

Intc ..ational application No.
PCT/KR 99/00282

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Group I: claims 1-3 and 7, drawn to a DNA construct comprising a first sequence coding for a purF gene and a second sequence coding for an antimicrobial peptide (claims 1-3) and production method of antimicrobial peptides (claim7)

Group II: claims 4-6, drawn to a special expression vector which is not characterized in a suitable manner nor referred to any of claims 1-3 or 7 in such a way that the expression vector of claims 4-6 and the DNA construct of claims 1-3 comprise a single inventive concept.

Group III: claim 8, drawn to a DNA construct, characterized by features, only,which do not link this construct with any of the subject matters of the previous claims as to form a single inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-3 and 7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00282

Although only a part of the sequence listing could be read- maybe lack of compatibility of the computer system.